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(71) Applicant *(for all designated States except US)*: UNIVERSITY OF MARYLAND BIOTECHNOLOGY INSTITUTE [US/US]; 4321 Hartwick Road, College Park, MD 20740 (US).

(72) Inventors; and
(75) Inventors/Applicants *(for US only)*: GALLO, Robert, C. [US/US]; 8513 Thomden Terrace, Bethesda, MD 02817 (US). BRYANT, Joseph [US/US]; 732 Ivy League Lane, Rockville, MD 20850 (US). LUNARDI-ISKANDAR, Yanto [FR/US]; 226 Lee Street, Gaithersburg, MD 20877 (US).

(74) Agents: ANTLER, Adriane, M. et al.; Pennie & Edmonds LLP, 1155 Avenue of the Americas, New York, NY 10036 (US).

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(54) Title: METHODS OF PROMOTING HEMATOPOIESIS USING DERIVATIVES OF HUMAN CHORIONIC GONADOTROPIN

(57) Abstract

The present invention relates to methods of treating or preventing diseases or disorders associated with hematopoietic deficiency by administration of human chorionic gonadotropin, β-human chorionic gonadotropin, a peptide containing a sequence of one or more portions of β-human chorionic gonadotropin, or fractions of a source of native human chorionic gonadotropin or native β-human chorionic gonadotropin. The invention also relates to methods of treating and preventing diseases or disorders associated with hematopoietic deficiency by administration of hematopoietic cells, the numbers of which have been increased by contacting the cells by administration of a therapeutic of the invention. Pharmaceutical compositions and methods of administration are also provided.

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METHODS OF PROMOTING HEMATOPOIESIS USING DERIVATIVES OF HUMAN CHORIONIC GONADOTROPIN

1. CROSS REFERENCE TO RELATED APPLICATION

This application is a continuation in part of co-pending application Serial No. 08/709,924, filed September 9, 1996, which is a continuation in part of co-pending Serial No. 08/669,654, filed June 24, 1996, both of which are incorporated by reference herein in their entireties.

2. FIELD OF THE INVENTION

The present invention is directed to methods of promoting hematopoiesis using human chorionic gonadotropin, fractions of preparations containing native human chorionic gonadotropin and native β-human chorionic gonadotropin, the β-chain of human chorionic gonadotropin and peptides consisting of a sequence of one or more portions of the β-chain of human chorionic gonadotropin. The invention provides for methods of increasing production of hematopoietic cells *in vitro* and *in vivo* and methods for treating diseases and disorders associated with a reduction in hematopoietic cell numbers. The invention also provides pharmaceutical compositions and methods of administration.

3. BACKGROUND OF THE INVENTION

3.1. HEMATOPOIETIC CELL PRODUCTION

The morphologically recognizable and functionally capable cells circulating in blood include erythrocytes, neutrophilic, eosinophilic, and basophilic granulocytes, B-, T-, non B-, non T-lymphocytes, and platelets. These mature hematopoietic cells derive from and are replaced, on demand, by morphologically recognizable dividing precursor cells for the respective lineages such as erythroblasts for the erythrocyte series, myeloblasts, promyelocytes and myelocytes for the granulocyte series, and megakaryocytes for the platelets. The precursor cells derive from more primitive cells that can simplistically be divided into two major

subgroups: stem cells and progenitor cells (for review, see Broxmeyer, H.E., 1983, "Colony Assays of Hematopoietic Progenitor Cells and Correlations to Clinical Situations," *CRC Critical Reviews in Oncology/Hematology* 1:227-257). The definitions of stem and progenitor cells are operational and depend on functional, rather than on morphological, criteria. Stem cells have extensive self-renewal or self-maintenance capacity (Lajtha, L.G., 1979, *Differentiation* 14:23), a necessity since absence or depletion of these cells could result in the complete depletion of one or more cell lineages, events that would lead within a short time to disease and death. Some of the stem cells differentiate upon need, but some stem cells or their daughter cells produce other stem cells to maintain the precious pool of these cells. Thus, in addition to maintaining their own kind, pluripotential stem cells are capable of differentiation into several sub-lines of progenitor cells with more limited self-renewal capacity or no self-renewal capacity. These progenitor cells ultimately give rise to the morphologically recognizable precursor cells. The progenitor cells are capable of proliferating and differentiating along one, or more than one, of the myeloid differentiation pathways (Lajtha, L.G. (Rapporteur), 1979, *Blood Cells* 5:447).

A variety of infectious agents, genetic abnormalities and environmental factors can cause a deficiency in one or more hematopoietic cell types. For example, hematological abnormalities have been observed in HIV-1 infected individuals (the human immunodeficiency virus (HIV) has been implicated as the primary cause of the slowly degenerative immune system disease termed acquired immune deficiency syndrome (AIDS) (Barre-Sinoussi, F., et al., 1983, *Science* 220:868-870; Gallo, R., et al., 1984, *Science* 224:500-503)) , particularly in the late stages of disease (Lunardi-Iskandar, Y. et al., 1989, *J. Clin. Invest* 83:610-615). These abnormalities include a reduction in $CD4^+$ T cells as well as cytopenias of one or more hematopoietic lineages, often associated with bone marrow morphologic abnormalities and

deficient progenitor cell growth (Lunardi-Iskandar, Y. et al., 1989, *J. Clin. Invest* 83:610-615; Louache, F. et al., 1992, *Blood* 180:2991-2999). Idiopathic thrombocytopenic purpura (ITP), characterized by significant reduction in platelet numbers, often afflicts subjects infected with HIV (Ballem, P.J. et al., 1992, *N. Engl. J. Med.* 327:1779). The destruction of platelets in sufferers of ITP appears to be mediated by platelet associated autoantibodies (Berchtold, P. and Wenger, M., 1993, *Blood* 81:1246; Ballem, P.J. et al., 1987, *J. Clin. Invest.* 80:33). Thus, because management of ITP generally involves immunosuppression, treatment of ITP in HIV infected patients is complicated as administration of immunosuppressive drugs is extremely detrimental in HIV infection.

Additionally, chemotherapy and radiation therapy used in the treatment of cancer and certain immunological disorders can cause pancytopenias or combinations of anemia, neutropenia and thrombocytopenia. Thus, the increase or replacement of hematopoietic cells is often crucial to the success of such treatments. (For a general discussion of hematological disorders and their causes, *see, e.g.*, "Hematology" *in* Scientific American Medicine, E. Rubenstein and D. Federman, eds., Volume 2, chapter 5, Scientific American, New York (1996)).

Furthermore, aplastic anemia presents a serious clinical condition as the overall mortality of all patients with aplastic anemias, in the absence of stem cell therapy, is high. Approximately 60-75% of individuals suffering from the disorder die within 12 months, in the absence of new stem cells. The overall incidence of these diseases is approximately 25 new cases per million persons per year. Although it is extremely unlikely that a single pathogenic mechanism accounts for all aplastic anemias, it is clear that provision of new hematopoietic stem cells is usually sufficient to allow permanent recovery, since transplantation of patients with aplastic anemia with bone marrow obtained from identical twins (*i.e.*, syngeneic) (Pillow, R.P., et al.,

1966, *N. Engl. J. Med.* 275:94-97) or from HLA-identical siblings (*i.e.*, allogeneic) (Thomas, E.D., et al., Feb. 5, 1972, *The Lancet*, pp. 284-289) can fully correct the disease. However, some patients with aplastic anemia reject the transplanted marrow. This complication is particularly common among patients who have been immunologically sensitized as a result of multiple therapeutic blood transfusions.

The current therapy available for many hematological disorders as well as the destruction of the endogenous hematopoietic cells caused by chemotherapy or radiotherapy is bone marrow transplantation. However, use of bone marrow transplantation is severely restricted since it is extremely rare to have perfectly matched (genetically identical) donors, except in cases where an identical twin is available or where bone marrow cells of a patient in remission are stored in a viable frozen state. Except in such autologous cases, there is an inevitable genetic mismatch of some degree, which entails serious and sometimes lethal complications. These complications are two-fold. First, the patient is usually immunologically incapacitated by drugs beforehand, in order to avoid immune rejection of the foreign bone marrow cells (host versus graft reaction). Second, when and if the donated bone marrow cells become established, they can attack the patient (graft versus host disease), who is recognized as foreign. Even with closely matched family donors, these complications of partial mismatching are the cause of substantial mortality and morbidity directly due to bone marrow transplantation from a genetically different individual.

Peripheral blood has also been investigated as a source of stem cells for hematopoietic reconstitution (Nothdurtt, W., et al., 1977, *Scand. J. Haematol.* 19:470-481; Sarpel, S.C., et al., 1979, *Exp. Hematol.* 7:113-120; Ragharachar, A., et al., 1983, *J. Cell. Biochem. Suppl.* 7A:78; Juttner, C.A., et al., 1985, *Brit. J. Haematol.* 61:739-745; Abrams, R.A., et al., 1983, *J. Cell. Biochem. Suppl.* 7A:53; Prummer, O., et

al., 1985, *Exp. Hematol.* 13:891-898). In some studies, promising results have been obtained for patients with various leukemias (Reiffers, J., et al., 1986, *Exp. Hematol.* 14:312-315; Goldman, J.M., et al., 1980, *Br. J. Haematol.* 45:223-231; Tilly, H., et al., July 19, 1986, *The Lancet*, pp. 154-155; see also To, L.B. and Juttner, C.A., 1987, *Brit. J. Haematol.* 66: 285-288, and references cited therein); and with lymphoma (Korbling, M., et al., 1986, *Blood* 67:529-532). Other studies using peripheral blood, however, have failed to effect reconstitution (Hershko, C., et al., 1979, *The Lancet* 1:945-947; Ochs, H.D., et al., 1981, *Pediatr. Res.* 15:601). Studies have also investigated the use of fetal liver cell transplantation (Cain, G.R., et al., 1986, *Transplantation* 41:32-25; Ochs, H.D., et al., 1981, *Pediatr. Res.* 15:601; Paige, C.J., et al., 1981, *J. Exp. Med.* 153:154-165; Touraine, J.L., 1980, *Excerpta Med.* 514:277; Touraine, J.L., 1983, *Birth Defects* 19:139; see also Good, R.A., et al., 1983, *Cellular Immunol.* 82:44-45 and references cited therein) or neonatal spleen cell transplantation (Yunis, E.J., et al., 1974, *Proc. Natl. Acad. Sci. U.S.A.* 72:4100) as stem cell sources for hematopoietic reconstitution. Cells of neonatal thymus have also been transplanted in immune reconstitution experiments (Vickery, A.C., et al., 1983, *J. Parasitol.* 69(3):478-485; Hirokawa, K., et al., 1982, *Clin. Immunol. Immunopathol.* 22:297-304).

Clearly, there is a tremendous need for methods of expanding blood cells *in vitro* or therapies which increase the production of hematopoietic cells *in vivo*.

3.2. HUMAN CHORIONIC GONADOTROPIN

Human chorionic gonadotropin (hCG), which is required for the maintenance of pregnancy, is a member of the glycoprotein hormone family. The glycoprotein hormones, which also include follicle-stimulating hormone (FSH), luteinizing hormone (LH) and thyroid-stimulating hormone (TSH), consist of two sub-units, α and β. These subunits are non-covalently linked to form a heterodimer, and heterodimer

formation has been shown to be required for receptor binding. Within a particular species, the α-subunits are identical among the glycoprotein hormones while the β-subunits differ and determine the receptor binding specificity of the particular hormone (Kornyei, J.L., et al., 1993, *Biol. Reprod.* 49:1149). The β-subunits of the glycoprotein hormones exhibit a high degree of sequence similarity within the N-terminal 114 amino acids. LH is the most similar to hCG with 85% sequence homology within the first 114 amino acids, and both proteins bind the same receptor. hCG, however, contains a C-terminal extension not present in the other glycoprotein β-chains (Lapthorn, A.J., et al., 1994, *Science* 369:455-461).

From the three dimensional crystal structure of hCG, it was determined that hCG, like the growth factors nerve growth factor (NGF), transforming growth factor-β (TGF-β) and platelet derived growth factor-β (PDGF-β), is a cysteine-knot glycoprotein. Proteins containing such a cysteine-knot motif have at least three disulfide bridges, two of which join adjacent anti-parallel strands of the peptide, thus, forming a ring, and one of which joins the peptide chain through the ring. Particular structures in the hCG β-chain include the determinant loop sequence (β93-100) which has been implicated in subunit association and the longest inter-cysteine loop (β38-57) which may play a role in receptor binding. Residues 47-53 appear to be exposed at the surface of this inter-cysteine loop (Lapthorn et al., 1994, *Nature* 369:455-461).

Harris, P.J. (1995, *The Lancet* 346:118-119) found that administration of hCG preparations to certain HIV infected subjects led to, among other improvements in symptoms of AIDS, increases in $CD4^+$ and $CD8^+$ T cell lymphocytes.

Citation of references hereinabove shall not be construed as an admission that such references are prior art to the present invention.

4. SUMMARY OF THE INVENTION

The present invention relates to therapeutic methods and compositions for treatment and prevention of diseases and disorders in which an increase in one or more types of hematopoietic cells is desirable. The therapeutic compounds of the invention are hCG and β-hCG preparations, therapeutically and prophylactically effective fractions of a source of native hCG or native β-hCG and therapeutically and prophylactically effective proteins containing a sequence of one or more portions (*i.e.*, a fusion protein comprising more than one β-hCG peptide sequence either as non-contigous or contiguous sequences, *e.g.*, having an amino acid sequence of one β-hCG peptide linked via a peptide bond to another β-hCG peptide) of β-hCG, and related derivatives and analogs. The present invention also relates to use of certain fractions (*i.e.* components of a source of hCG or β-hCG (preferably native hCG or β-hCG, *i.e.* not recombinantly produced) isolated away from other components in the source of hCG or β-hCG by a separation technique known in the art) of any source of hCG or β-hCG, such as commercial hCG preparations and human (preferably early, *i.e.*, first trimester) pregnancy urine, which fractions have anti-HIV and/or anti-Kaposi's Sarcoma activity and/or pro-hematopoietic activity.

The invention provides for treatment and prevention of diseases and disorders (*e.g.*, involving hematopoietic cell deficiencies) by administration either of a therapeutic compound of the invention or of hematopoietic cells, the numbers of which have been increased *in vitro* by contact with a therapeutic compound of the invention. The invention thus also provides *in vitro* methods of expanding hematopoietic cells. The therapeutic compounds of the invention include: hCG, β-hCG, therapeutically and prophylactically effective fractions of a source of native hCG or native β-hCG, therapeutically and prophylactically effective peptides having a sequence of one or more portions of β-hCG, modified derivatives of hCG, β-hCG and β-hCG peptides, and nucleic acids encoding β-hCG and therapeutically and prophylactically

effective peptides having a sequence of one or more portions of β-hCG, and derivatives and analogs of the foregoing.

In a specific embodiment, gene therapy methods are provided using hCG and β-hCG preparations, therapeutically and prophylactically effective fractions of a source of hCG or β-hCG and therapeutically and prophylactically effective proteins containing a sequence of one or more portions of β-hCG, and related derivatives and analogs to induce proliferation of hematopoietic progenitor or stem cells into which cells a nucleic acid of interest is introduced either before or after proliferation. The proliferation induced by the methods of the invention can be with or without concomitant hematopoietic cell differentiation, and, in a preferred embodiment, is proliferation followed by differentiation of the cells.

The invention further provides assays, both *in vitro* and *in vivo*, for testing the efficacy of the Therapeutics of the invention.

The invention also provides methods of administration and pharmaceutical compositions containing a Therapeutic of the invention.

4.1. DEFINITIONS AND ABBREVIATIONS

As used herein, the following abbreviations will have the meanings indicated:

AIDS = Acquired Immune Deficiency Syndrome

ARC = AIDS Related Complex

BFU-E = burst forming unit-erythroid. A hematopoietic progenitor cell which is capable of producing a colony of erythroid progeny cells in semi-solid medium.

CFU = colony forming unit. A cell which is capable of producing a colony of progeny cells in semi-solid medium.

CFU-GEMM = colony forming unit-granulocyte, erythrocyte, monocyte/macrophage, megakaryocyte. A multipotential hematopoietic progenitor cell which is capable of producing a colony composed of granulocyte, erythrocyte,

monocyte/macrophage, and megakaryocyte progeny in semi-solid medium.

CFU-GM = colony forming unit-granulocyte, macrophage. A hematopoietic progenitor cell which is capable of producing a colony composed of granulocyte and macrophage progeny in semi-solid medium.

CFU-MK = colony forming unit-megakaryocyte. A hematopoietic progenitor cell which is capable of producing a colony composed of megakaryocyte progeny in semi-solid medium.

CFU-S = colony forming unit-spleen. A multipotential stem cell with self-renewal capacity, which, upon inoculation into a lethally irradiated mouse, is capable of producing a colony (module) on the spleen. CFU-S is not a marrow-repopulating cell; it is a less primitive stem cell which does not provide long-term engraftment in an animal.

CSF = colony stimulating factor

Epo = erythropoietin

FBS = fetal bovine serum. Also known as fetal calf serum.

G-CSF = granulocyte colony stimulating factor

GM-CSF = granulocyte-macrophage colony stimulating factor

hCG = Human Chorionic Gonadotropin

HIV = Human Immunodeficiency Virus

ITP = Idiopathic thrombocytopenic purpura (a severe platelet deficiency)

PB = peripheral blood

PBMC = Peripheral Blood Mononuclear Cells

5. DESCRIPTION OF THE FIGURES

Figures 1A-D. Effects of an hCG preparation on indicators of SIV infection in SIV-infected macaques. SIV was given intravenously at a dose of $10^{4.5}$ $TCID_{50}$ per ml. (A) SIV titer was monitored over time in months by quantifying the p27 gag protein (Organon Teknika assay) as nanograms (ng) of p27/ml of plasma from the plasma of the SIV infected

macaques. Treated SIV-infected macaques (indicated as Rx) were given hCG APL, 3000 IU, 2x weekly. Plasma levels of p27 gag in these treated monkeys are indicated on the graph by lines with diamonds, number (#) signs or filled circles. Results with the untreated SIV-infected macaques (indicated UnRx) are indicated by the lines with either stars or triangles. (B) CD4+ T cell levels were determined in cells/mm^3 in SIV-infected macaques either treated with hCG or untreated over time in months. Results from the SIV-infected monkeys treated with hCG (APL) (Rx) are indicated by lines with diamonds, number (#) signs or filled circles, while results with the untreated monkeys (UnRx) are indicated by lines with stars or triangles. (C) Change in weight in kilograms (kg) was monitored in treated and untreated SIV-infected monkeys over time in months. Weight changes in the SIV-infected monkeys treated with hCG (APL) (Rx) are indicated by lines with diamonds, # signs or filled circles, while results in the untreated monkeys (UnRx) are indicated by lines with stars or triangles. (D) Levels of CD4+ T cells were monitored in normal uninfected monkeys either treated with hCG (APL) or untreated over time in months. CD4+ T cell levels in the untreated monkeys are indicated by lines with sun-like figures or squares, and the results in the treated monkeys are indicated by lines with pentagonal figures or with filled inverted triangles.

Figures 2A-J. Effects of administration of hCG preparations on HIV-1 viral load and CD4+ T cell levels in individual patients in the clinical study described in Section 7.2 *infra*. Figures A and B are data from patient PHOJ, C and D from patient PG1, E and F from patient PG3, G and H from patient PHVE, and I and J from patient PG17. In panels A, C, E, G and I, viral load and CD4+ T Cell counts are plotted over time (in months). Viral load (measured by RT-PCR in panels A and G and by the Roche Amplicor test in panels C, E and I) is plotted as the logarithm of the viral load (represented by line with "X" data points). The CD4+ T Cell levels are plotted as CD4+ T Cells/ml (represented by

line with triangle data points). Panels B, D, F, H, and J plot the dosage of hCG in IU (X 1000) per week over time in months, with the timing of other therapies indicated above the graph with a thick arrow.

Figures 3A-C. These bar graphs demonstrate the effects of hCG preparations and peptides on hematopoiesis *in vitro*. (A) This bar graph depicts results of colony assays in terms of number of colonies for CFU-MIX (colony forming units of megakaryocytes, erythrocytes, granulocytes and monocytes). (B) This bar graph presents data from colony assays for BFU-e (Burst forming units of erythrocytes) in terms of number of colonies. (C) This bar graph presents results from colony assays of CFU-GM (colony forming units of granulo-macrophages) in terms of number of colonies. For all three graphs, results are shown for cells isolated from cord blood ("cord") and bone marrow ("marrow"). The results are averages of 3 sets of results with less than 10% variation and are representative of multiple experiments. The results from no treatment are indicated by open bars; the results with α-hCG are represented by solid bars; the results with hCG-APL (hCGapl) are represented by bars with a lattice pattern; the results with native β-hCG preparation (NbhCG) are represented by cross-hatched bars; the results with the highly purified hCG preparation (CR127) are represented by open bars; the results with the β-hCG peptide of amino acids 109-119 (SEQ ID NO:7) (b109-119) are shown by the diagonally stippled bars; the results with the β-hCG peptide of amino acids 45-57 (SEQ ID NO:6) (b45-57) are shown by the bars with the diamonds; the results with the circularized β-hCG peptide of amino acids 44-57 (SEQ ID NO:12) with cysteine substituted for the amino acid at position 44 (b45-57c) are represented by the diagonally striped bars; and the results with the mixture of scrambled β-hCG peptides of amino acids 45-57 and 109-119 (bmix45+109) are represented by the vertically striped bars.

Figure 4. Nucleotide (SEQ ID NO:1) and amino acid (SEQ ID NO:2) sequences of β-hCG.

Figures 5A and B. Schematic depiction of the structures of (A) the linear peptide of amino acids 45-57 (SEQ ID NO:6) of the β-hCG sequence depicted in Figure 4 (SEQ ID NO:2) where the amino acid residues at positions 47 and 51 are substituted by a branch made up of diaminobutyric acid peptide bonded to proline, and (B) the circularized peptide of amino acids 44-57 (SEQ ID NO:12) with valine at position 44 substituted with cysteine, which protein is circularized via a disulfide bond between its amino- and carboxy-terminal cysteines. In both A and B, amino acids are represented by their three letter amino acid code, except for the branched residues and the terminal cysteines, for which the structure is depicted.

Figures 6A-F. These graphs depict results from the fractionation by SUPERDEX™ 200 gel filtration of a commercial hCG preparation APL™ (Wyeth Ayerst) and early pregnancy urine. (A) and (D). These graphs depicts the relative amount of protein in mg/ml in each fraction identified by fraction number in the hCG APL™ fractionation (A) and early pregnancy urine fractionation (D). The fractions containing the hCG dimer and β-core protein are identified with arrows and the labels "hCG" and "β-core" respectively. (B) and (E). These graphs present the percent inhibition of growth of cultured KS cells by the individual fractions from the hCG APL™ (B) and early pregnancy urine (E) using KS cell clonogenic assays. The results are plotted as percent inhibition versus fraction number. (C) and (F). These graphs depict the effect of the hCG APL™ (C) and early pregnancy urine (F) fractions on HIV replication in vitro. Specifically, this graph presents data on the percentage inhibition of HIV-1 IIIB viral infection of PBMCs as a function of fraction number.

Figures 7A-C. These bar graphs demonstrate the effects of hCG preparations, fractions and peptides on hematopoiesis *in vitro*. (A) Results of colony assays in terms of percent increase of hematopoiesis for CFU-GEMM (colony forming units of megakaryocytes, erythrocytes, granulocytes and monocytes). (B) Data from colony assays for BFU-e (Burst forming units of

erythrocytes) in terms of percent increase of hematopoiesis. (C) Results from colony assays of CFU-GM (colony forming units of granulo-macrophages) in terms of percent increase of hematopoiesis. In all three graphs, bar 1 represents results from treatment with PBS alone; bar 2, the results with 100 μg/ml α-hCG; bar 3, with 200 IU/ml APL™ hCG; bar 4, 200 IU/ml of the highly purified hCG preparation CR 127; bar 5, 100 μg/ml native β-hCG preparation; bar 6, 100 μg/ml of the circularized β-hCG peptide of amino acids 44-57 with cysteine substituted for the amino acid at position 44 (SEQ ID NO:26); bar 7, 100 μl/ml of fraction 65 of the hCG APL™ fractionation; bars 8 and 9, 100 μl/ml of fractions 65 and 26, respectively, of the early pregnancy urine fractionation; and bar 10, 100 μg/ml of the β-hCG core protein.

Figures 8A-C. Graphs of change in viral load and CD4⁺ T cell levels with hCG therapy. (A) The change in viral load is plotted as the logarithm of viral load after therapy ("Logload") as a function of viral load before therapy ("Baselog"). (B) The change in CD4⁺ T cell levels is plotted as CD4⁺ T cell levels after therapy (in CD4⁺ T cells/ml) ("CD4") as a function of CD4⁺ T cell levels before therapy (in CD4⁺ T cells/ml) ("CD4Base"). (C) Plot of linear regression analysis of the change in viral load ("vlchange") as a function of weekly dose of hCG in IU ("HCGIU"). For all three panels, data points for patients on hCG therapy as well as non-protease and protease inhibitors are represented by open triangles, those on hCG therapy and non-protease inhibitors by open diamonds, and those on hCG alone by solid circles.

Figures 9A and B. (A) Plot of protein concentration (as mAUFS, milli absorbance units, at 280 nm) as a function of the fraction number of the hCG APL™ preparation SUPERDEX™ 200 fractionation. (B) Plot of protein concentration (as mAUFS, milli absorbance units, at 280 nm) of molecular weight markers of 670 kD, 158 kD, 44 kD, 17 kD and 1.3 kD (as indicated above the plot) as a function of fraction number of

a SUPERDEX™ 200 column run under the same conditions as the fractionation plotted in panel A.

Figures 10A-E. Mass spectrometry profiles of fractions 61, 63, 64, 65, and 67 in panels A-E, respectively.

6. DETAILED DESCRIPTION OF THE INVENTION

The present invention relates to therapeutic methods and compositions for treatment and prevention of diseases and disorders in which increased amounts of hematopoietic cells are desirable (*e.g.*, disorders associated with reduced numbers of one or more hematopoietic cell types) by administration of hCG, β-hCG and therapeutically or prophylactically effective proteins (*e.g.*, peptides) having a sequence of one or more portions of β-hCG (β-hCG peptides), and derivatives and analogs thereof, and therapeutically or prophylactically effective fractions of a source of native hCG or native β-hCG (*i.e.* from a naturally occurring source and not hCG or β-hCG which has been recombinantly produced). The invention provides for treatment and prevention of hematopoietic cell deficiencies by administration either of a therapeutic compound of the invention or of hematopoietic cells, the numbers of which have been increased *in vitro* by contact with a therapeutic compound of the invention. The invention also provides methods for expansion of hematopoietic cells *in vitro* by contact with a Therapeutic of the invention. The therapeutic compounds of the invention include, but are not limited to: hCG, β-hCG, therapeutically and prophylactically effective fractions of a source of native hCG or native β-hCG, and therapeutically and prophylactically effective β-hCG peptides, *i.e.*, those fractions and peptides which prevent or treat HIV infection (*e.g.*, as demonstrated in *in vitro* and *in vivo* assays described *infra*), and derivatives and analogs thereof, as well as nucleic acids encoding hCG, β-hCG and therapeutically and prophylactically effective β-hCG peptides and derivatives and analogs thereof (*e.g.*, for use in gene therapy).

In a preferred embodiment, a therapeutic composition of the invention comprises a β-hCG peptide, the amino acid sequence of which consists of amino acid numbers 41-53, 42-53, 43-53, 44-53, 44-57, 45-53, 46-53, 45-54, 45-55, 45-56, 45-58, 47-54, 47-55, 47-56, 47-58, 48-145, 58-145, 109-145, 7-40, 46-65, or 48-56 (SEQ ID NOS:8-25 or 33-35, respectively) of Figure 4 (a portion of SEQ ID NO:2), particularly a β-hCG peptide which consists of amino acid numbers 41-54, 45-54 or 109-119 (SEQ ID NOS:3, 4, or 7, respectively), most preferably of a β-hCG peptide which consists of amino acids 47-53 (SEQ ID NO:5) or 45-57 (SEQ ID NO:6).

In other preferred embodiments, the therapeutic comprises a β-hCG peptide, the amino acid sequence of which consists of circularized (via a disulfide bond between its amino- and carboxy- terminal cysteines) 44-57 (SEQ ID NO:26) with the valine at position 44 substituted with cysteine ((Val44Cys) 45-57 circularized) (depicted in Figure 5B), 45-57 (SEQ ID NO:6) where the amino acid residues at positions 47 and 51 are substituted by a branch, where the branches are made up of diaminobutyric acid peptide bonded to a proline residue (depicted in Figure 5A).

In another embodiment, a protein is used which contains the amino acid sequence of two or more peptides of different portions of the β-hCG sequence (either as non-contiguous or contiguous sequenced), *e.g.*, in which the N-terminus of one portion is linked to the C-terminus of another portion by peptide bond(s). In a specific embodiment, a protein is used, the amino acid sequence of which consists of amino acids 45-57 (SEQ ID NO:6) linked at the C-terminus via a peptide bond to the N-terminus of amino acids 109-119 (SEQ ID NO:7) or linked at the N-terminus via a peptide bond to the C-terminus of amino acids 110-119 (SEQ ID NO:27); or a protein is used that has an amino acid sequence of amino acids 47-57 (SEQ ID NO:28) linked at the C-terminus via a peptide bond to the N-terminus of amino acids 108-119 (SEQ ID NO:29) of the β-hCG sequence; *i.e.*, the fused peptides

represented as 45-57::109-119, 110-119::45-57, or 47-57::108-119 (SEQ ID NOS:30-32, respectively). In yet another embodiment a protein is used which is a cyclic, fused peptide, particularly a cyclic, fused peptide having a sequence consisting of β-hCG amino acids 110-119 (SEQ ID NO:27) linked at the C-terminus via a peptide bond to the N-terminus of amino acids 45-57 (SEQ ID NO:6) and being circularized by a disulfide bond between the terminal cysteines at positions 110 and 57.

In another embodiment, a protein is used that (a) comprises a β-hCG amino acid sequence consisting of amino acid numbers 41-54, 45-54, 47-53, 45-57, 109-119, 41-53, 42-53, 43-53, 44-53, 44-57, 45-53, 46-53, 45-54, 45-55, 45-56, 45-58, 47-54, 47-55, 47-56, 47-58, 48-145, 58-145, 109-119, 109-145, 7-40, 46-65 or 48-56 (SEQ ID NOS:3-25 or 33-35, respectively) as depicted in Figure 4 (a portion of SEQ ID NO:2) and (b) lacks β-hCG amino acids contiguous to said sequence. The amino acid sequence of β-hCG is depicted in Figure 4 (SEQ ID NO:2).

For clarity of disclosure, and not by way of limitation, the detailed description of the invention is divided into the subsections which follow.

6.1. THERAPEUTIC USES

The invention provides for treatment or prevention of diseases and disorders in which increased numbers of one or more hematopoietic cell types are desirable (*e.g.*, diseases or disorders associated with one or more hematopoietic cell deficiencies) by administration of a therapeutic compound (termed herein "Therapeutic") of the invention or by administration of hematopoietic cells, the production of which has been induced *in vitro* by contacting the cells with a Therapeutic of the invention. Such "Therapeutics" include but are not limited to: hCG, β-hCG and derivatives thereof, and therapeutically or prophylactically effective fractions of a source of native hCG or native β-hCG and therapeutically or prophylactically effective β-hCG peptides, *i.e.*, those

fraction and peptides which prevent or treat hematopoietic deficiencies (e.g., as demonstrated in *in vitro* and *in vivo* assays described *infra*) as well as modifications, derivatives and analogs thereof and nucleic acids encoding hCG, β-hCG and therapeutically and prophylactically effective β-hCG peptides, and derivatives and analogs thereof. In one embodiment, the Therapeutic of the invention is a protein containing an amino acid sequence of a therapeutically and prophylactically effective portion or portions of β-hCG.

In a preferred embodiment, the Therapeutic of the invention is a protein having a sequence of amino acid numbers 41-54, 45-54, 47-53 or 45-57 (SEQ ID NOS:3-6, respectively) of the β-hCG sequence depicted in Figure 4 (a portion of SEQ ID NO:2). In other embodiments, the Therapeutic of the invention is a protein having a sequence of amino acid numbers 41-53, 42-53, 43-53, 44-53, 44-57, 45-53, 46-53, 45-54, 45-55, 45-56, 45-58, 47-54, 47-55, 47-56, 47-58, 48-145, 58-145, 109-145, 7-40, 46-65, or 48-56 (SEQ ID NOS:8-25 or 33-35, respectively) of the β-hCG sequence of Figure 4 (a portion of SEQ ID NO:2). Additionally, the present inventors have found that different preparations of hCG and β-hCG have variable effects on hematopoietic cell proliferation *in vitro* and *in vivo*. Specifically, the inventors found that among the (non-recombinant) commercial preparations they investigated, hCG from Fujisawa was the most effective, hCG from APL (Wyeth-Ayerst) the next most effective, and pregnyl (Organon) the next most effective. hCG preparations and fractions of hCG and β-hCG preparations can be screened for utility in inducing hematopoiesis *in vitro* or *in vivo* by the methods described *infra* in Section 6.4 or any method known in the art.

In one embodiment of the invention, the Therapeutic is administered directly to a patient suffering from a disease or disorder amenable to treatment by increasing production of one or more hematopoietic cell types (e.g., a disease or disorder associated with a hematopoietic cell deficiency). In another embodiment of the invention, hematopoietic cells,

preferably stem and/or progenitor cells, are obtained, contacted with a Therapeutic of the invention *in vitro* to induce proliferation of the cells, and then administered to a subject suffering from a disease or disorder associated with a hematopoietic cell deficiency. Preferably, autologous hematopoietic cells (obtained from the subject or its identical twin) are reintroduced into the subject after *in vitro* expansion. In this embodiment, gene therapy methods can be performed by introducing a nucleic acid of interest, e.g., containing a gene which provides a function desired in a subject, into the hematopoietic cells, before or after expansion of the cells by contact with a Therapeutic. Hematopoietic cell subpopulations can be isolated for use, before or after expansion *in vitro*. For example, blood cells can be isolated and expanded, and optionally also differentiated, *in vitro*, followed by introduction of all or a portion of the cells (e.g, purified platelets, red blood cells, lymphocytes, etc.) into a patient.

In general, disorders that can be treated by methods of the invention include, but are not limited to, five broad categories. First are diseases resulting from a failure or dysfunction of normal blood cell production and maturation (i.e., aplastic anemia, cytopenias and hypoproliferative stem cell disorders). The second group are neoplastic, malignant diseases in the hematopoietic organs (e.g., leukemia and lymphomas). The third group of disorders comprises those of patients with a broad spectrum of malignant solid tumors of non-hematopoietic origin. Induction of hematopoietic cell proliferation or administration of replacement hematopoietic cells in these patients serves as a bone marrow rescue procedure, which is provided to a patient following otherwise lethal chemotherapy or irradiation of the malignant tumor. The fourth group of diseases consists of autoimmune conditions, where the hematopoietic cells serve as a source of replacement of an abnormal immune system. The fifth group of diseases comprises a number of genetic disorders which can be corrected by infusion of hematopoietic stem cells,

preferably syngeneic, which prior to transplantation have undergone gene therapy. Particular diseases and disorders which can be treated by induction of hematopoietic cell production *in vivo* or by administration of hematopoietic cells expanded *in vitro* include but are not limited to those listed in Table 1, and described *infra*.

TABLE 1

DISEASES OR DISORDERS WHICH CAN BE TREATED BY INCREASING PRODUCTION OF HEMATOPOIETIC CELLS

I. Diseases resulting from a failure or dysfunction of normal blood cell production and maturation
- hyperproliferative stem cell disorders
- aplastic anemia
- pancytopenia
- agranulocytosis
- thrombocytopenia
- red cell aplasia
- Blackfan-Diamond syndrome
- due to drugs, radiation, or infection
- idiopathic

II. Hematopoietic malignancies
- acute lymphoblastic (lymphocytic) leukemia
- chronic lymphocytic leukemia
- acute myelogenous leukemia
- chronic myelogenous leukemia
- acute malignant myelosclerosis
- multiple myeloma
- polycythemia vera
- agnogenic myelometaplasia
- Waldenstrom's macroglobulinemia
- Hodgkin's lymphoma
- non-Hodgkin's lymphoma

III. Immunosuppression in patients with malignant, solid tumors
- malignant melanoma
- carcinoma of the stomach
- ovarian carcinoma
- breast carcinoma
- small cell lung carcinoma
- retinoblastoma
- testicular carcinoma
- glioblastoma
- rhabdomyosarcoma
- neuroblastoma
- Ewing's sarcoma
- lymphoma

IV. Autoimmune diseases
- rheumatoid arthritis
- diabetes type I
- chronic hepatitis
- multiple sclerosis
- systemic lupus erythematosus

V. Genetic (congenital) disorders
- anemias
- familial aplastic
- Fanconi's syndrome
- Bloom's syndrome
- pure red cell aplasia (PRCA)
- dyskeratosis congenita
- Blackfan-Diamond syndrome
- congenital dyserythropoietic syndromes I-IV
- Chwachmann-Diamond syndrome
- dihydrofolate reductase deficiencies
- formamino transferase deficiency
- Lesch-Nyhan syndrome
- congenital spherocytosis
- congenital elliptocytosis
- congenital stomatocytosis
- congenital Rh null disease
- paroxysmal nocturnal hemoglobinuria
- G6PD (glucose-6-phosphate dehydrogenase) variants 1,2,3
- pyruvate kinase deficiency
- congenital erythropoietin sensitivity deficiency
- sickle cell disease and trait
- thalassemia alpha, beta, gamma
- met-hemoglobinemia
- congenital disorders of immunity
- severe combined immunodeficiency disease (SCID)
- bare lymphocyte syndrome
- ionophore-responsive combined immunodeficiency
- combined immunodeficiency with a capping abnormality
- nucleoside phosphorylase deficiency
- granulocyte actin deficiency
- infantile agranulocytosis
- Gaucher's disease
- adenosine deaminase deficiency
- Kostmann's syndrome
- reticular dysgenesis
- congenital leukocyte dysfunction syndromes

VI. Others
- osteopetrosis
- myelosclerosis
- acquired hemolytic anemias
- acquired immunodeficiencies

infectious disorders causing primary or secondary immunodeficiencies
bacterial infections (e.g., Brucellosis, Listeriosis, tuberculosis, leprosy)
parasitic infections (e.g., malaria, Leishmaniasis)
fungal infections
disorders involving disproportions in lymphoid cell sets and impaired immune functions due to aging
phagocyte disorders
Kostmann's agranulocytosis
chronic granulomatous disease
Chediak-Higachi syndrome
neutrophil actin deficiency
neutrophil membrane GP-180 deficiency
metabolic storage diseases
mucopolysaccharidoses
mucolipidoses
miscellaneous disorders involving immune mechanisms
Wiskott-Aldrich Syndrome
alpha 1-antitrypsin deficiency

6.1.1. DISEASES RESULTING FROM A FAILURE OR DYSFUNCTION OF NORMAL BLOOD CELL PRODUCTION AND MATURATION

In a preferred aspect, a Therapeutic of the invention is used to treat a disease resulting from a failure or dysfunction of normal blood cell production and maturation, such as an aplastic anemia, a cytopenia or a hypoproliferative stem cell disorder. These disorders entail failure of stem cells in bone marrow to provide normal numbers of functional blood cells. The aplastic anemias result from the failure of stem cells to give rise to the intermediate and mature forms of red cells, white cells, and platelets. While red cell production is usually most seriously affected, a marked decrease in production of other mature blood cell elements is also seen as some anemias specifically affect production of white cells and/or platelets. The large majority of these anemias are acquired during adult life, and do not have any apparent genetic predisposition. About half of these acquired anemias arise in the absence of any obvious causative factor such as

exposure to poisons, drugs or disease processes that impair stem cell function; these are termed idiopathic aplastic anemias. The remaining cases are associated with exposure to an extremely diverse array of chemicals and drugs and also occur as the consequence of viral infections, such as HIV infection, and after pregnancy. Other specific types of aplastic anemia are termed agranulocytosis or thrombocytopenia to indicate that the major deficiency lies in particular white cells or in platelet production, respectively. These non red blood cell deficiencies are also often associated with HIV infection. Also significantly associated with HIV infection is a severe platelet deficiency, Idiopathic Thrombocytopenic Purpura (ITP). Additionally, agranulocytosis may be associated with autoimmune syndromes such as systemic lupus erythematosus (SLE) or with other infections, such as neonatal rubella.

In addition, immune deficiencies which are the primary or secondary result of infection by pathogenic microorganisms can be treated by administration of a Therapeutic of the invention. For example, immune deficiencies caused by microorganisms which are intracellular pathogens of hematopoietic cells, can be treated by the provision of new hematopoietic cells. These new hematopoietic cells can be generated by contacting hematopoietic stem and/or progenitor cells *in vitro* with a Therapeutic of the invention to cause proliferation of the cells. Microorganisms causing immune deficiencies which may be treated according to this embodiment of the invention include but are not limited to gram-negative bacilli such as *Brucella* or *Listeria*, the mycobacterium which are the etiological agents of tuberculosis or of Hansen's disease (leprosy), parasites such as *Plasmodium* (the etiological agents of malaria) or *Leishmania*, and fungi (such as those that cause pneumonia and other lethal infections secondary to immunodeficiencies) (for a discussion of many of these disorders, see Harrison's Principles of Internal Medicine, 1970, 6th Edition, Wintrobe, M.M., et al., eds., McGraw-Hill, New York, pp. 798-1044).

In a preferred embodiment of the invention, a Therapeutic of the invention is administered for the treatment of a cytopenia associated with HIV infection. The hematopoietic deficiencies associated with HIV infection include reduction in $CD4^+$ T cells and other lymphocytes, red blood cells, platelets, specifically ITP, and neutrophils. Such a disorder is treated by contacting hematopoietic stem and/or progenitor cells *in vitro* with a Therapeutic of the invention and then infusing the resulting hematopoietic cells into the subject in need of treatment. In a another preferred embodiment, the disorder is treated by direct administration of a Therapeutic of the invention to the subject in need of treatment. Assays for determining the efficacy of particular Therapeutics for treatment of hematopoietic deficiencies associated with HIV infection are detailed in Section 6.4 *infra*.

6.1.2. TREATMENT OF MALIGNANCIES

Hyperproliferative malignant stem cell disorders as well as non-hematopoietic malignancies can be treated with chemotherapy or radiation therapy along with rescue of hematopoietic cells by direct administration of a Therapeutic of the invention or by administration of hematopoietic cells induced to proliferate by contacting the cells with a Therapeutic of the invention. The conditions that can be treated according to the invention include, but are not limited to, the leukemias listed in Table 1 and the solid tumors listed in Table 1.

These malignancies are currently treated by, *inter alia*, chemotherapy and, when feasible, allogeneic bone marrow transplantation. However, allogeneic HLA identical sibling bone marrow is available only to less than one-third of patients, and this treatment is associated with transplantation-related complications such as immunodeficiency and graft versus host disease. Induction of hematopoietic cell proliferation *in vivo* or provision of autologous hematopoietic stem and progenitor cells expanded

by administration of a Therapeutic *in vitro* permits hematopoietic reconstitution of patients lacking suitable allogeneic donors and eliminates the risks of graft versus host disease arising from allogeneic marrow transplantation. Thus, in a specific embodiment, a Therapeutic is used to induce proliferation in hematopoietic cells which are then administered to a patient who has undergone chemotherapy or radiation therapy for treatment of cancer or an immunological disorder. In another embodiment, a Therapeutic is directly administered to a patient who has undergone chemotherapy or radiation therapy for treatment of cancer or an immunological disorder.

6.1.3. AUTOIMMUNE DISORDERS

Many chronic inflammatory and degenerative diseases are characterized by a continuous immune reaction against the body's own tissues. Such autoimmune disorders include but are not limited to rheumatoid arthritis and other inflammatory osteopathies, diabetes type I, chronic hepatitis, multiple sclerosis, and systemic lupus erythematosus. Autoimmune disorders are often treated by lymphoid irradiation. Administration of a Therapeutic of the invention or of cells produced by exposure to a Therapeutic *in vitro* can be valuable to repopulate the hematopoietic system after radiotherapy.

Anti-inflammatory drugs such as steroids retard the inflammatory cells which are activated by autoreactive T cells, but do not prevent T cells which recognize self-proteins from activating new inflammatory cells. A more direct approach to treating autoimmune diseases depends on eradication of T cells by irradiation of the lymphoid tissues, and relying on stem cells from the unirradiated bone marrow to repopulate the patient's hematopoietic system. The rationale is that the formation of new populations of mature T cells from bone marrow stem cells may result in absence of T cells that have reactivity to self-specific antigens. This procedure, called total lymphoid irradiation (TLI), has been

used to treat intractable rheumatoid arthritis (Strober, S., et al., 1985, *Annals of Internal Medicine* 102:441-449, 450-458). These clinical trials showed that in the majority of otherwise intractable cases, joint disease was significantly alleviated for at least 2-3 years. However, the major drawback to such treatment is failure of stem cells in the bone marrow of these elderly patients to efficiently repopulate the hematopoietic system, resulting in infections and bleeding disorders. Analogous studies have been made of the effects of TLI as an alternative to cytotoxic drugs for treatment of SLE (Strober, S., et al., 1985, *Ann. Internal Med.* 102:450). Studies of the use of TLI to treat intractable SLE have also shown that this treatment alleviates disease activity, but is severely limited by failure of bone marrow stem cells to rapidly and efficiently repopulate the hematopoietic system after irradiation.

Thus, a Therapeutic of the invention can be administered to promote proliferation of the remaining hematopoietic cells to increase the success of TLI therapy. Additionally, hematopoietic stem and progenitor cells can be isolated from the patient before treatment, induced to proliferate *in vitro* and then introduced into the patient after TLI treatment to repopulate the hematopoietic system.

6.1.4. GENE THERAPY

Administration of hematopoietic, preferably hematopoietic stem and progenitor, cells which have been induced to proliferate with a Therapeutic of the invention and have undergone gene therapy, *i.e.*, which have stably incorporated a heterologous gene capable of expression by their progeny cells, can be of great value in the treatment of diseases and disorders affecting cells of hematopoietic lineage. In one embodiment, hematopoietic reconstitution with such recombinant hematopoietic cells can be used in the treatment of genetic disorders of the hematopoietic system. Such genetic disorders include but are not limited to those listed in Table 1, *supra*. Genetic deficiencies or

dysfunctions of hematopoietic cells can be treated by supplying, to a patient, recombinant stem and progenitor cells. In a specific embodiment, patients who have hematopoietic cells which lack a gene or have a mutant gene, can be provided stem and progenitor cells that have incorporated a functional counterpart of the deficient gene. In particular, such genes which can be subject to gene therapy include but are not limited to hemoglobin or enzymes which mediate its synthetic pathway (*e.g.*, for treatment of anemias such as beta-thalassemia, sickle-cell disease).

In another specific embodiment, patients with infections by pathogenic microorganisms which occur in or affect a hematopoietic cell lineage can be treated with recombinant hematopoietic cells. Such recombinant hematopoietic cells can contain a heterologous gene which is expressed as a product which ameliorates disease symptoms, is toxic to the pathogen without significant detriment to the host, or interferes with the pathogen's life cycle, etc. Pathogens which cause infections which may be treated with recombinant stem cells according to this embodiment of the invention include but are not limited to lymphotropic viruses such as HIV; gram-negative bacilli such as *Brucella* or *Listeria*; the mycobacterium which cause tuberculosis, or which cause Hansen's disease (leprosy); parasites such as *Plasmodium* (the etiological agents of malaria), or *Leishmania*; and fungi (such as those that cause pneumonia and other lethal infections secondary to immunodeficiencies) (for a discussion of many of these disorders, see Harrison's Principles of Internal Medicine, 1970, 6th Edition, Wintrobe, M.M., et al., eds., McGraw-Hill, New York, pp. 798-1044).

As a particular embodiment, it is possible to construct recombinant stem or progenitor cells that express a sequence which is "anti-sense" to the nucleic acid of a hematopoietic cell pathogen. Such a sequence, which is complementary to the pathogen's RNA or DNA, can hybridize to and inactivate such RNA or DNA, inhibiting the function or expression of the nucleic acid and disrupting the pathogen's life cycle. As a

particular example, recombinant hematopoietic cells can be used in the treatment of AIDS. Recombinant stem and progenitor cells which express an anti-sense nucleic acid that is complementary to a critical region (*e.g.*, the long-terminal repeat or polymerase sequence) of the HIV genome (Wain-Hobson et al., 1985, *Cell* 40:9-17) can be used for hematopoietic reconstitution for the treatment of AIDS.

Many methods of gene therapy are available in the art (for general reviews of the methods of gene therapy, see Goldspiel et al., 1993, *Clinical Pharmacy* 12:488-505; Wu and Wu, 1991, *Biotherapy* 3:87-95; Tolstoshev, 1993, *Ann. Rev. Pharmacol. Toxicol.* 32:573-596; Mulligan, 1993, *Science* 260:926-932; and Morgan and Anderson, 1993, *Ann. Rev. Biochem.* 62:191-217; May, 1993, *TIBTECH* 11(5):155-215). Methods commonly known in the art of recombinant DNA technology which can be used are described in Ausubel et al. (eds.), 1993, Current Protocols in Molecular Biology, John Wiley & Sons, NY; and Kriegler, 1990, Gene Transfer and Expression, A Laboratory Manual, Stockton Press, NY.

In a preferred aspect, the nucleic acid which provides a gene product desired in a subject is introduced into an expression vector that produces the gene product. In particular, such a nucleic acid has a promoter operably linked to the nucleic acid sequence of interest, said promoter being inducible or constitutive, and, optionally, tissue-specific. In another particular embodiment, a nucleic acid molecule is used in which the sequences of interest are flanked by regions that promote homologous recombination at a desired site in the genome, thus providing for intrachromosomal expression of the desired protein (Koller and Smithies, 1989, *Proc. Natl. Acad. Sci. USA* 86:8932-8935; Zijlstra et al., 1989, *Nature* 342:435-438).

In an embodiment of the invention, the nucleic acid is introduced into a hematopoietic cell that is then expanded by exposure to a Therapeutic of the invention prior to administration *in vivo* of the resulting recombinant cell. Alternatively, the nucleic acid can be introduced after

expansion. Such introduction can be carried out by any method known in the art, including, but not limited to, transfection, electroporation, microinjection, infection with a viral vector containing the nucleic acid sequences, cell fusion, chromosome-mediated gene transfer, microcell-mediated gene transfer, spheroplast fusion, etc. Numerous techniques are known in the art for the introduction of foreign genes into cells (*see, e.g.*, Loeffler and Behr, 1993, *Meth. Enzymol.* 217:599-618; Cohen et al., 1993, *Meth. Enzymol.* 217:618-644; Cline, 1985, *Pharmac. Ther.* 29:69-92) and may be used to construct the recombinant hematopoietic cells for purposes of gene therapy. The technique should provide for the stable transfer of the nucleic acid to the cell, so that the nucleic acid is expressible by the cell and preferably heritable and expressible by its cell progeny.

The resulting recombinant cells can be delivered to a patient by various methods known in the art. In a preferred embodiment, recombinant hematopoietic cells are administered intravenously. The amount of cells envisioned for use depends on the desired effect, patient state, etc., and can be determined by one skilled in the art.

6.2. PREPARATION OF HEMATOPOIETIC CELLS *IN VITRO*

Sources of hematopoietic stem and progenitor cells, which cells can be induced to proliferate according to one embodiment of the present invention, include but are not limited to bone marrow, fetal and neonatal blood (preferably from the umbilical cord and/or placenta), fetal liver, adult peripheral blood, neonatal thymus, and neonatal spleen. The foregoing list of sources is deemed to include cell samples (*e.g.*, cryopreserved cells, cell lines, long-term cell cultures) derived therefrom. The source is mammalian, *e.g.*, mouse, cow, horse, primate, monkey, and is most preferably human. Techniques for obtaining such stem and progenitor cells are well known in the art. For example, in one particular embodiment, human bone marrow cells can be obtained from the posterior iliac crest by needle aspiration

(see, e.g., Kodo et al., 1984, *J. Clin. Invest.* 73:1377-1384). Neonatal blood can be obtained at birth by direct drainage from the umbilical cord and/or by needle aspiration from the delivered placenta at the root and at distended veins (see U.S. Patent Nos. 5,004,681 and 5,192,553). Fetal blood can be obtained, e.g., by taking it from the fetal circulation at the placental root with the use of a needle guided by ultrasound (Daffos et al., 1985, *Am. J. Obstet. Gynecol.* 153:655-660; Daffos et al., 1983, *Am. J. Obstet. Gynecol.* 146:985), by placentocentesis (Valenti, 1973, *Am. J. Obstet. Gynecol.* 115:851; Cao et al., 1982, *J. Med. Genet.* 19:81), by fetoscopy (Rodeck, C.H., 1984, in *Prenatal Diagnosis*, Rodeck, C.H. and Nicolaides, K.H., eds., Royal College of Obstetricians and Gynaecologists, London), etc.

The method of the invention which comprises contacting hematopoietic stem and/or progenitor cells (or other hematopoietic cells) with a Therapeutic of the invention, can be carried out on unseparated, partially separated, or purified cell populations, before and/or after cryopreservation (and thawing) or *in vitro* culturing of such cell populations, before and/or after introduction of a recombinant gene, and any other desired manipulations of the cells. In a preferred aspect, samples (e.g. bone marrow or adult blood or neonatal blood) can be subjected to physical and/or immunological cell separation procedures so as to enrich for hematopoietic stem and progenitor cells (e.g., prior to culturing in the presence of a Therapeutic of the invention to induce proliferation of the cells).

Various procedures are known in the art and can be used to enrich for stem and progenitor cells. These include but are not limited to equilibrium density centrifugation, velocity sedimentation at unit gravity, immune rosetting and immune adherence, counterflow centrifugal elutriation, T lymphocyte depletion, and fluorescence-activated cell sorting, alone or in combination. Procedures have been reported for the isolation of very highly enriched

populations of stem/progenitor cells. U.S. Patent No. 5,061,620 dated October 29, 1991 discloses a method for isolation of human hematopoietic stem cells. Murine CFU-S have been purified by several groups using slightly different procedures (Visser et al., 1984, *J. Exp. Med.* 59:1576; Nijhof et al., 1984, *Exp. Cell Res.* 155:583; Bauman et al., 1986, *J. Cell. Physiol.* 128:133; Lord and Spooncer, 1986, *Lymphokine Res.* 5:59). Studies using human (Emerson et al., 1985, *J. Clin. Invest.* 76:1286) or murine (Nicola et al., 1981, *Blood* 58:376) fetal liver cells have yielded highly enriched progenitor cells with up to 90% of them being colony forming cells for multi-, erythroid-, and granulocyte-macrophage lineages. CFU-E have also been very highly enriched (Nijhof et al., 1983, *J. Cell Biol.* 96:386). Purification of adult mouse marrow CFU-GM with cloning efficiencies of up to 99% in semi-solid medium has been accomplished by pretreatment of mice three days prior to sacrifice with cyclophosphamide, density separation of cells on Ficoll-Hypaque, and counterflow centrifugal elutriation (Williams et al., 1987, *Exp. Hematol.* 15:243). The resulting fraction of cells contained no detectable CFU-GEMM, BFU-E or CFU-MK, but up to 10% of the cells formed CFU-S measured at day 12. These procedures, or modifications thereof, can be used.

Human stem and progenitor cells are present in the non-adherent, low density, T-lymphocyte-depleted fraction of bone marrow, spleen, and adult and cord blood cells. Low density (density less than 1.077 gm/cm^3) cells can be separated by use of Ficoll-Hypaque (Pharmacia Fine Chemicals, Piscataway, NJ) or Percol (Broxmeyer, H.E., 1982, *J. Clin. Invest.* 69:632-642). In this procedure, the mature cells of the granulocytic series, which are not needed for transplantation, are removed in the dense fraction which goes to the bottom of the tube. An adherence/nonadherence separation protocol can also be used for enrichment of hematopoietic stem and progenitor cells.

It is also possible to use cell separation procedures that entail immunological recognition of cells. Stem and

progenitor cells can be isolated by positive or negative selection using antibodies that recognize antigenic determinants on the surface of cells. One means is to separate the cells by using monoclonal antibodies which recognize cell surface determinants on these cells, in conjunction with separation procedures such as fluorescence-activated cell sorting or panning (Broxmeyer et al., 1984, *J. Clin. Invest.* 73:939-953). Human hematopoietic stem and progenitor cells contain antigenic determinants that are not present on all other cells, which can be used in antibody selection protocols for enrichment purposes; such antigens include but are not limited to those described *infra*.

Within the human system, several antigens have been found on stem/progenitor cells. The first antigenic system studied intensively was that of the MHC class II antigens, especially HLA-DR. This antigen has been found on CFU-GEMM, BFU-E, and CFU-GM (Lu et al., 1983, *Blood* 61:250; Winchester et al., 1977, *Proc. Natl. Acad. Sci. U.S.A.* 74:4012; Busch et al., 1987, *Blut* 54:179). Several investigators have suggested that HLA-DR are not found, or are present at a low density on cells earlier than CFU-GEMM (Moore et al., 1980, *Blood* 55:682; Keating et al., 1984, *Blood* 64:1159).

Groups of antibodies have been used to distinguish different progenitors of the granulocyte-macrophage lineage (Ferrero et al., 1983, *Proc. Natl. Acad. Sci. U.S.A.* 80:4114). Type 1 CFU-GM contribute all of the peripheral blood CFU-GM, as well as a small number of bone marrow CFU-GM. They express surface antigens recognized by S3-13 and S17-25 antibodies, but not by R1B19 and WGHS-29-1 antibodies. Type 2 CFU-GM are present only in the marrow and react with S3-13, R1B19, and WGHS-29-1. Culture of type 1 CFU-GM in liquid culture generates type 2 CFU-GM. These antibodies have also been used to characterize CFU-GM from patients with chronic myeloproliferative disorders (Robak et al., 1985, *Leukemia Res.* 9:1023; Ferrero et al., 1986, *Cancer Res.* 46:975).

Other antigens on human stem/progenitor cells include those reactive with the My10 (Leary et al., 1987, *Blood* 69:953; Strauss et al., 1986, *Exp. Hematol.* 14:879), 3C5 (Katz et al., 1985, *Leukemia Res.* 9:191; Katz et al., 1986, *Leukemia Res.* 10:961), RFB-1 (Bodger et al., 1983, *Blood* 61:1006), 12-8 (Andrews et al., 1986, *Blood* 67:842), and L4F3 (Andrews et al., 1986, *Blood* 68:1030) antibodies. The antigen recognized by L4F3 is on CFU-GM, CFU-MK, BFU-E, and CFU-GEMM, but is apparently absent from cells which generate these progenitors in suspension culture (*id.*). The antigen recognized by the My10 antibody is CD34 (Civin et al., U.S. Patent No. 4,714,680 dated December 22, 1987.) Two subsets of pluripotent hematopoietic stem cells have been reported, a $CD34^+$ $HLA\text{-}DR^+$ $CD38^-$ subset and a more primitive $CD34^+$ HLA-DR $CD38^-$ subset, with a gradual increase in CD38 expression as the hematopoietic cells proceed toward a more differentiated state (Huang and Terstappen, 1992, *Nature* 360:745-749; Terstappen et al., 1992, *Leukemia* 6:993-1000). The antigen recognized by another antibody, My11, is expressed on CFU-GM, but not on BFU-E or CFU-GEMM (Strauss et al., 1986, *Exp. Hematol.* 14:935). Receptors for various lectins are also expressed on stem/progenitor cells (Nicola et al., 1980, *J. Cell Physiol.* 103:217; Reisner et al., 1982, *Blood* 59:360; Reisner et al., 1978, *Proc. Natl. Acad. Sci. U.S.A.* 75:2933; Aizawa and Tavassoli, 1986, *Int. J. Cell Cloning* 4:464).

To expand the numbers of the hematopoietic stem and/or progenitor cells, the hematopoietic stem and/or progenitor cells (or precursor cells thereof) are exposed to or contacted with a composition comprising a Therapeutic of the invention for a sufficient time period, *i.e.*, until the desired number of cells is obtained and the time period should be for as long as it is desired to keep cells self-renewing. Preferably, the cells are contacted with the Therapeutic, for example but not limited to, 200 IU/ml hCG (*e.g.*, hCG APL) or β-hCG preparation or a fraction of a source of hCG or β-hCG or 100 μg/ml β-hCG peptide, preferably a β-hCG peptide having the amino acid sequence of amino acid

numbers 45-57 or 109-119 (SEQ ID NOS:6 or 7, respectively), or circularized peptide of amino acid numbers 44-57 (SEQ ID NO:12) with cysteine substituted for valine at position 44, or a branched peptide of amino acid numbers 45-57 (SEQ ID NO:6) with diaminobutyric acid substituted for the amino acids at positions 47 and 51 with proline peptide bonded to the diaminobutyric acid residues, or a circularized branched peptide of amino acids 44-57 (SEQ ID NO:12) with cysteine substituted for valine at position 44 and with diaminobutyric acid substituted for the amino acids at positions 47 and 51 with proline peptide bonded to the diaminobutyric acid residues or a peptide having the sequence of amino acids 45-57 (SEQ ID NO:6) linked at the C-terminus via a peptide bond to the N-terminus of amino acids 109-119 (SEQ ID NO:7) or linked at the N-terminus via a peptide bond to the C-terminus of amino acids 110-119 (SEQ ID NO:27); or an amino acid sequence of amino acids 47-57 (SEQ ID NO:28) linked at the C-terminus via a peptide bond to the N-terminus of amino acids 108-119 (SEQ ID NO:29) or the cyclic, fused peptide, having a sequence of amino acids 110-119 (SEQ ID NO:27) linked at the C-terminus via a peptide bond to the N-terminus of amino acids 45-57 (SEQ ID NO:6) and being circularized by a disulfide bond between the terminal cysteines at positions 110 and 57 , where all amino acid numbers are of the β-hCG sequence depicted in Figure 4 (a portion of SEQ ID NO:2), while under appropriate culture conditions, for a time period in the range of 1-21 or, more preferably, 7-21 days.

The composition comprising the Therapeutic of the invention, to which the stem and progenitor cells are exposed according to the invention, optionally also contains other growth factors and/or cytokines or cell culture materials, including but not limited to erythropoietin (Epo), Steel factor (SLF), IL-1, IL-3, IL-4, IL-6, IL-11, G-CSF, GM-CSF, FBS, adult PB plasma, alone or in combination. Preferably, factors are present that cause proliferation or, less preferably, differentiation of cells that are CFU-GEMM or earlier cells, *e.g.*, IL-3, GM-CSF.

Contacting of the stem and progenitor cells with the Therapeutic preferably occurs during cell culture and thus, the Therapeutic is preferably added to the cell culture medium being used to culture the hematopoietic stem and/or progenitor cells. Such culturing can be by any method known in the art, including, but not limited to, cells grown in culture dishes, test tubes, roller bottles, bioreactors (perfusion system machines wherein cells are grown on a surface with continual perfusion by medium; *e.g.*, as sold by Aastrom Biosciences, Inc., Ann Arbor, MI), etc. Various protocols have been described for the growth *in vitro* of cord blood or bone marrow cells, and it is envisioned that such procedures, or modifications thereof, may be employed (*see, e.g.* Smith, S. and Broxmeyer, H.E., 1986, *Br. J. Haematol.* 63:29-34; Dexter et al., 1977, *J. Cell. Physiol.* 91:335; Witlock and Witte, 1982, *Proc. Natl. Acad. Sci. U.S.A.* 79:3608-3612). The cell culture medium is supplemented to contain an effective concentration of the Therapeutic, for example but not limited to, 200 I.U. hCG (APL) or β-hCG preparation or 100 μg/ml of a β-hCG peptide or a fraction of a native hCG or native β-hCG preparation.

Progeny cells of hematopoietic stem and progenitor cells of can be generated in vitro; the differentiated progeny cells thus generated can be therapeutically useful. For example, in one embodiment of this aspect of the invention, hematopoietic stem cells and/or CFU-GEMM progenitor cells, can be induced to differentiate into platelets. Such platelets can be used, for example, for infusion into a patient with thrombocytopenia, such as, but not limited to, the ITP associated with HIV infection. In another embodiment, granulocytes can be generated *in vitro* prior to infusion into a patient. One or more of the hematopoietic progeny cells can be generated *in vitro*, allowing for the *in vitro* production of blood components. In one embodiment, the generation of differentiated blood components is accompanied by expansion of the hematopoietic stem and progenitor cell pool, in order to allow for production of a greater quantity

of differentiated cells. Various growth factors can be used to promote expansion and/or differentiation of hematopoietic stem and progenitor cells, such as cytokines (growth factors) including, but not limited to, G-CSF, CSF-1, IL-3, IL-5, tumor necrosis factor-β, and α-interferon. The blood components which are thus produced have uses which are not limited to therapeutic uses *in vivo*. For example, such progeny cells can be used *in vitro*, *e.g.*, for the production and isolation of hematopoietic cell products such as growth factors, antibodies, etc.

A specific embodiment of the invention relates to a method of increasing the amount of hematopoietic cells, which method comprises contacting *in vitro* a non-terminally differentiated hematopoietic cell with a composition comprising an amount of a Therapeutic of the invention effective to increase proliferation of the cell, under conditions suitable and for a time period sufficient to increase the numbers of said hematopoietic cell. For example, hematopoietic cell numbers can be increased by contacting a non-terminally differentiated hematopoietic cell (*e.g.*, a cell isolated from bone marrow or blood, adult or fetal or umbilical cord blood) with a composition comprising 200 IU/ml hCG (*e.g.*, hCG APL) or β-hCG preparation or a fraction of a source of hCG or β-hCG or 100 μg/ml β-hCG peptide, preferably a β-hCG peptide having the amino acid sequence of amino acid numbers 45-57 or 109-119 (SEQ ID NOS:6 or 7, respectively), or circularized peptide of amino acid numbers 44-57 (SEQ ID NO:12) with cysteine substituted for valine at position 44, or a branched peptide of amino acid numbers 45-57 (SEQ ID NO:6) with diaminobutyric acid substituted for the amino acids at positions 47 and 51 with proline peptide bonded to the diaminobutyric acid residues, or a circularized branched peptide of amino acids 44-57 (SEQ ID NO:12) with cysteine substituted for valine at position 44 and with diaminobutyric acid substituted for the amino acids at positions 47 and 51 with proline peptide bonded to the diaminobutyric acid residues or a peptide having the sequence

of amino acids 45-57 (SEQ ID NO:6) linked at the C-terminus via a peptide bond to the N-terminus of amino acids 109-119 (SEQ ID NO:7) or linked at the N-terminus via a peptide bond to the C-terminus of amino acids 110-119 (SEQ ID NO:27); or an amino acid sequence of amino acids 47-57 (SEQ ID NO:28) linked at the C-terminus via a peptide bond to the N-terminus of amino acids 108-119 (SEQ ID NO:29) or the cyclic, fused peptide, having a sequence of amino acids 110-119 (SEQ ID NO:27) linked at the C-terminus via a peptide bond to the N-terminus of amino acids 45-57 (SEQ ID NO:6) and being circularized by a disulfide bond between the terminal cysteines at positions 110 and 57, where all amino acid numbers are of the β-hCG sequence depicted in Figure 4 (a portion of SEQ ID NO:2), and culturing the cell for at least ten days.

6.3. hCG, β-hCG AND β-hCG PEPTIDES AND DERIVATIVES THEREOF

In a specific embodiment, a preparation comprising hCG is used that contains not only the hCG heterodimer but also peptide fragments thereof, e.g., β chain peptides.

In a preferred embodiment of the invention, proteins (e.g., peptides), the amino acid sequence of which consists of one or more portions effective to increase the production of one or more hematopoietic cell types of the β-hCG sequence (β-hCG peptides) are used to treat or prevent hematopoietic deficiencies. In various specific embodiments, the portion(s) of the β-hCG sequence are at least 3, 5, 10, 20, or 30 amino acids. These proteins are preferably β-hCG peptides, or nucleic acids encoding β-hCG peptides, from amino acids 41-54, 45-54, 47-53 and 45-57 (SEQ ID NOS:3-6, respectively) of Figure 4 (a portion of SEQ ID NO:2). In other embodiments, these proteins are β-hCG peptides, or nucleic acids encoding β-hCG peptides, of amino acids 41-53, 42-53, 43-53, 44-53, 44-57, 45-53, 46-53, 45-54, 45-55, 45-56, 45-58, 47-54, 47-55, 47-56, 47-58, 48-145, 58-145, 109-145, 7-40, 46-65, or 48-56 (SEQ ID NOS:8-25 or 33-35, respectively) of Figure 4 (a portion of SEQ ID NO:2), and

preferably containing a sequence from amino acids 41-54, 45-54 or 109-119 (SEQ ID NOS:3, 4 or 7, respectively) of Figure 4 (a portion of SEQ ID NO:2), and most preferably containing a sequence from amino acids 47-53 or 45-57 of Figure 4 (a portion of SEQ ID NO:2), or circular [C44V]45-57 peptide (SEQ ID NO:26), or branched 45-57 (SEQ ID NO:6) peptide, or branched circular [V44C]45-57 peptide are used to treat or prevent hematopoietic deficiencies.

In another embodiment, the invention provides proteins, the amino acid sequences of which consist of two or more at least 5, 7 or 10 amino acid, non-naturally contiguous portions of the β-hCG sequence (Figure 4 (SEQ ID NO:2)) linked by peptide bonds between the N-terminus of one portion and the C-terminus of another portion. Specifically, proteins, the amino acid sequences of which consist of amino acids 45-57 (SEQ ID NO:6) linked at the C-terminus via a peptide bond to the N-terminus of a peptide of amino acids 109-119 (SEQ ID NO:7) or linked at the N-terminus via a peptide bond to the C-terminus of amino acids 110-119 (SEQ ID NO:27); or an isolated protein of amino acids 47-57 (SEQ ID NO:28) linked at the C-terminus via a peptide bond to the N-terminus of amino acids 108-119 (SEQ ID NO:29) of the β-hCG sequence depicted in Figure 4 (portions of SEQ ID NO:2), *i.e.*, the fused peptides denoted as 45-57::109-119, 110-119::45-57, or 47-57::108-119 (SEQ ID NOS:30-32, respectively). Derivatives of the foregoing fusion proteins are also provided (*e.g.*, branched, cyclized, N- or C-terminal chemically modified, etc.). In another embodiment, fusion proteins comprising two or more such portions of the β-hCG sequence are provided; such portions may or may not be contiguous to one another (*i.e.*, an intervening sequence may be present). Molecules comprising such portions linked by hydrocarbon linkages are also provided. In another embodiment, the peptides of the invention (i) have an amino acid sequence consisting of no more than 8 peptides of the β-hCG sequence (Figure 4 (SEQ ID NO:2)) and (ii) comprise amino

acid numbers 47-53 (SEQ ID NO:5) of β-hCG (Figure 4 (SEQ ID NO:2)).

In another embodiment, a protein is used that (a) comprises one or more portions of the amino acid sequence consisting of β-hCG, a peptide having an amino acid sequence consisting of said portion(s) being effective to increase production of one or more hematopoietic cell types; and (b) lacks β-hCG amino acids contiguous to said portion(s). In another embodiment, a protein is used that (a) comprises a β-hCG amino acid sequence consisting of amino acid numbers 41-54, 45-54, 47-53, 45-57, 109-119, 41-53, 42-53, 43-53, 44-53, 44-57, 45-53, 46-53, 45-54, 45-55, 45-56, 45-58, 47-54, 47-55, 47-56, 47-58, 48-145, 58-145, 109-119, 109-145, 7-40, 46-65 or 48-56 (SEQ ID NOS:3-25 or 33-35, respectively) as depicted in Figure 4 (a portion of SEQ ID NO:2) and (b) lacks β-hCG amino acids contiguous to said sequence. Peptides containing the above sequences in which only conservative substitutions have been made are also provided by the present invention, as but one example of peptide derivatives within the scope of the invention. Analogs of the above-mentioned proteins and peptides which have one or more amino acid substitutions forming a branched peptide (*e.g.*, by substitution with an amino acid or amino acid analog having a free amino- or carboxy-side chain that forms a peptide bond with a sequence of one or more amino acids, including but not limited to prolines) or allowing circularization of the peptide (*e.g.*, by substitution with a cysteine, or insertion of a cysteine at the amino- or carboxy-terminus or internally), to provide a sulfhydryl group for disulfide bond formation, are also provided.

Other β-hCG peptides, and nucleic acids encoding these peptides, may have utility in the therapeutic methods of the invention. The utility of β-hCG peptides may be determined by the *in vitro* and *in vivo* assays described in Section 6.4 *infra* or by any other method known in the art.

In specific embodiments, peptides of less than 50, or less than 25, amino acids are provided.

The invention also relates to derivatives, modifications and analogs of β-hCG peptides. One embodiment of the invention provides a purified derivative of a protein effective to increase the production of one or more hematopoietic cell types, which protein contains an amino acid sequence of one or more portions effective to increase the production of one or more hematopoietic cell types of β-hCG. Another embodiment of the invention provides a purified derivative of a protein effective to increase the production of one or more hematopoietic cell types, the amino acid sequence of which protein is selected from the group consisting of amino acid numbers 41-54, 45-54, 47-53, 45-57, 109-119, 41-53, 42-53, 43-53, 44-53, 44-57, 45-53, 46-53, 45-54, 45-55, 45-56, 45-58, 47-54, 47-55, 47-56, 47-58, 48-145, 58-145, 109-145, 7-40, 46-65 or 48-56 (SEQ ID NOS:3-25 or 33-35, respectively) as depicted in Figure 4 (a portion of SEQ ID NO:2). In another embodiment, β-hCG peptide derivatives can be made by altering the β-hCG peptide sequence by substitutions, additions or deletions that provide for therapeutically effective molecules. Thus, the β-hCG peptide derivatives include peptides containing, as a primary amino acid sequence, all or part of the particular β-hCG peptide sequence including altered sequences in which functionally equivalent amino acid residues are substituted for residues within the sequence resulting in a peptide which is functionally active. For example, one or more amino acid residues within the sequence can be substituted by another amino acid of a similar polarity which acts as a functional equivalent, resulting in a silent alteration. Conservative substitutions for an amino acid within the sequence may be selected from other members of the class to which the amino acid belongs. For example, the nonpolar (hydrophobic) amino acids include alanine, leucine, isoleucine, valine, proline, phenylalanine, tryptophan and methionine. The polar neutral amino acids include glycine, serine, threonine, cysteine, tyrosine, asparagine, and glutamine. The positively charged (basic) amino acids include arginine, lysine and histidine.

The negatively charged (acidic) amino acids include aspartic acid and glutamic acid. Such β-hCG peptide derivatives can be made either by chemical peptide synthesis or by recombinant production from a nucleic acid encoding the β-hCG peptide which nucleic acid has been mutated. Any technique for mutagenesis known in the art can be used, including but not limited to, chemical mutagenesis, *in vitro* site-directed mutagenesis (Hutchinson, C., et al., 1978, *J. Biol. Chem* 253:6551), use of TAB® linkers (Pharmacia), etc.

In addition, β-hCG peptides and analogs and derivatives of β-hCG peptides can be chemically synthesized. (See, e.g., Merrifield, 1963, *J. Amer. Chem. Soc.* 85:2149-2156.) For example, peptides can be synthesized by solid phase techniques, cleaved from the resin, and purified by preparative high performance liquid chromatography (e.g., see Creighton, 1983, Proteins, Structures and Molecular Principles, W.H. Freeman and Co., N.Y., pp. 50-60). β-hCG peptides can also be synthesized by use of a peptide synthesizer. The composition of the synthetic peptides may be confirmed by amino acid analysis or sequencing (e.g., the Edman degradation procedure; see Creighton, 1983, Proteins, Structures and Molecular Principles, W.H. Freeman and Co., N.Y., pp. 34-49). Furthermore, if desired, nonclassical amino acids or chemical amino acid analogs can be introduced as a substitution or addition into the β-hCG peptide. Non-classical amino acids include but are not limited to the D-isomers of the common amino acids, 2,4-diaminobutyric acid, α-amino isobutyric acid, 4-aminobutyric acid, Abu, 2-amino butyric acid, γ-Abu, ε-Ahx, 6-amino hexanoic acid, Aib, 2-amino isobutyric acid, 3-amino propionic acid, ornithine, norleucine, norvaline, hydroxyproline, sarcosine, citrulline, homocitrulline, cysteic acid, t-butylglycine, t-butylalanine, phenylglycine, cyclohexylalanine, β-alanine, fluoro-amino acids, designer amino acids such as β-methyl amino acids, Cα-methyl amino acids, Nα-methyl amino acids, and amino acid analogs in general. Furthermore, the amino acid can be D (dextrorotary) or L (levorotary).

By way of example but not by way of limitation, peptides of the invention can be chemically synthesized and purified as follows: Peptides can be synthesized by employing the N-α-9-fluorenylmethyloxycarbonyl or Fmoc solid phase peptide synthesis chemistry using a Rainin Symphony Multiplex Peptide Synthesizer. The standard cycle used for coupling of an amino acid to the peptide-resin growing chain generally includes: (1) washing the peptide-resin three times for 30 seconds with N,N-dimethylformamide (DMF); (2) removing the Fmoc protective group on the amino terminus by deprotection with 20% piperdine in DMF by two washes for 15 minutes each, during which process mixing is effected by bubbling nitrogen through the reaction vessel for one second every 10 seconds to prevent peptide-resin settling; (3) washing the peptide-resin three times for 30 seconds with DMF; (4) coupling the amino acid to the peptide resin by addition of equal volumes of a 250 mM solution of the Fmoc derivative of the appropriate amino acid and an activator mix consisting or 400 mM N-methylmorpholine and 250 mM (2-(1H-benzotriazol-1-4))-1,1,3,3-tetramethyluronium hexafluorophosphate (HBTU) in DMF; (5) allowing the solution to mix for 45 minutes; and (6) washing the peptide-resin three times for 30 seconds of DMF. This cycle can be repeated as necessary with the appropriate amino acids in sequence to produce the desired peptide. Exceptions to this cycle program are amino acid couplings predicted to be difficult by nature of their hydrophobicity or predicted inclusion within a helical formation during synthesis. For these situations, the above cycle can be modified by repeating step 4 a second time immediately upon completion of the first 45 minute coupling step to "double couple" the amino acid of interest. Additionally, in the first coupling step in peptide synthesis, the resin can be allowed to swell for more efficient coupling by increasing the time of mixing in the initial DMF washes to three 15 minute washes rather than three 30 second washes. After peptide synthesis, the peptide can be cleaved from the resin as follows: (1) washing the peptide-resin three times for 30

seconds with DMF; (2) removing the Fmoc protective group on the amino terminus by washing two times for 15 minutes in 20% piperdine in DMF; (3) washing the peptide-resin three times for 30 seconds with DMF; and (4) mixing a cleavage cocktail consisting of 95% trifluoroacetic acid (TFA), 2.4% water, 2.4% phenol, and 0.2% triisopropysilane with the peptide-resin for two hours, then filtering the peptide in the cleavage cocktail away from the resin, and precipitating the peptide out of solution by addition of two volumes of ethyl ether. Specifically, to isolate the peptide, the ether-peptide solution can be allowed to sit at -20°C for 20 minutes, then centrifuged at 6,000xG for 5 minutes to pellet the peptide, and the peptide can be washed three times with ethyl ether to remove residual cleavage cocktail ingredients. The final peptide product can be purified by reversed phase high pressure liquid chromatography (RP-HPLC) with the primary solvent consisting of 0.1% TFA and the eluting buffer consisting of 80% acetonitrile and 0.1% TFA. The purified peptide can then be lyophilized to a powder.

In a preferred embodiment, the invention provides a peptide with branched amino acids (branched peptide), preferably a branched peptide of amino acids 45-57 (SEQ ID NO:6) with branches occurring at positions 47 and 51, respectively, instead of the Gly and Ala residues normally present. Most preferably, diaminobutyric acid is substituted for the gly and ala residues at positions 47 and 51, respectively, and proline bonded to both diaminobutyric acid residues (45-57 branched) as shown in Figure 5A.

In other specific embodiments, branched versions of the β-hCG peptides listed hereinabove are provided, *e.g.*, by substituting one or more amino acids within the β-hCG sequence with an amino acid or amino acid analog with a free side chain capable of forming a peptide bond with one or more amino acids (and thus capable of forming a "branch").

Branched peptides may be prepared by any method known in the art for covalently linking any naturally occurring or synthetic amino acid to any naturally occurring or synthetic

amino acid in a peptide chain which has a side chain group able to react with the amino or carboxyl group on the amino acids so as to become covalently attached to the peptide chain. In particular, amino acids with a free amino side chain group, such as, but not limited to, diaminobutyric acid, lysine, arginine, ornithine, diaminopropionic acid and citrulline, can be incorporated into a peptide so that an amino acid can form a branch therewith, for example, by forming a peptide bond to the free amino side group, from that residue. Alternatively, amino acids with a free carboxyl side chain group, such as, but not limited to, glutamic acid, aspartic acid and homocitrulline, can be incorporated into the peptide so that an amino acid can form a branch therewith, for example, by forming a peptide bond to the free carboxyl side group, from that residue. The amino acid forming the branch can be linked to a side chain group of an amino acid in the peptide chain by any type of covalent bond, including, but not limited to, peptide bonds, ester bonds and disulfide bonds. In a specific embodiment, amino acids, such as those described above, that are capable of forming a branch point, are substituted for β-hCG residues within a peptide having a β-hCG sequence.

Branched peptides can be prepared by any method known in the art. For example, but not by way of limitation, branched peptides can be prepared as follows: (1) the amino acid to be branched from the main peptide chain can be purchased as an N-α-tert-butyloxycarbonyl (Boc) protected amino acid pentafluorophenyl (Opfp) ester and the residue within the main chain to which this branched amino acid will be attached can be an N-Fmoc-α-γ-diaminobutyric acid; (2) the coupling of the Boc protected amino acid to diaminobutyric acid can be achieved by adding 5 grams of each precursor to a flask containing 150 ml DMF, along with 2.25 ml pyridine and 50 mg dimethylaminopyridine and allowing the solution to mix for 24 hours; (3) the peptide can then be extracted from the 150 ml coupling reaction by mixing the reaction with 400 ml dichlormethane (DCM) and 200 ml 0.12N HCl in a 1 liter

separatory funnel, and allowing the phases to separate, saving the bottom aqueous layer and re-extracting the top layer two more times with 200 ml 0.12 N HCl; (4) the solution containing the peptide can be dehydrated by adding 2-5 grams magnesium sulfate, filtering out the magnesium sulfate, and evaporating the remaining solution to a volume of about 2-5 ml; (5) the dipeptide can then be precipitated by addition of ethyl acetate and then 2 volumes of hexanes and then collected by filtration and washed two times with cold hexanes; and (6) the resulting filtrate can be lyophilized to achieve a light powder form of the desired dipeptide.

Branched peptides prepared by this method will have a substitution of diaminobutyric acid at the amino acid position which is branched. Branched peptides containing an amino acid or amino acid analog substitution other than diaminobutyric acid can be prepared analogously to the procedure described above, using the N-F-moc coupled form of the amino acid or amino acid analog.

In a preferred embodiment, the peptide is a cyclic peptide, preferably a cyclic peptide of β-hCG amino acids 44-57 (SEQ ID NO:26) with cysteine substituted for valine at position 44 and circularized via a disulfide bond between the cysteine residues at positions 44 and 57 (C[V44C] 45-57) (Figure 5B) or a cyclic fused peptide of β-hCG amino acids 110-119 (SEQ ID NO:27) linked at the C-terminus by a peptide bond to the N-terminus of amino acids 45-57 (SEQ ID NO:6) and circularized via a disulfide bond between the cysteine residues at positions 110 and 57. In another preferred embodiment, the peptide is a cyclic branched peptide of β-hCG amino acids 44-57 (SEQ ID NO:12) with cysteine substituted for valine at position 44 and circularized via a disulfide bond between the cysteine residues at positions 44 and 57 and positions 47 and 51 substituted with a diaminobutyric acid residue on which a proline is peptide bonded to its free amino sidechain.

Cyclization can be, for example, but not by way of limitation, via a disulfide bond between two cysteine

residues or via an amide linkage. For example, but not by way of limitation, disulfide bridge formation can be achieved by (1) dissolving the purified peptide at a concentration of between 0.1.-0.5 mg/ml in 0.01 M ammonium acetate, pH 7.5; (2) adding to the dissolved peptide 0.01 M potassium ferricyanide dropwise until the solution appears pale yellow in color and allowing this solution to mix for 24 hours; (3) concentrating the cyclized peptide to 5-10 ml of solution, repurifying the peptide by reverse phase-high pressure liquid chromatography (RP-HPLC) and finally lyophilizing the peptide. In a specific embodiment, in which the peptide does not contain two appropriately situated cysteine residues, cysteine residues can be introduced at the amino-terminus and/or carboxy-terminus and/or internally such that the peptide to be cyclized contains two cysteine residues spaced such that the residues can form a disulfide bridge. Alternatively, a cyclic peptide can be obtained by generating an amide linkage using, for example but not limited to, the following protocol: An allyl protected amino acid, such as aspartate, glutamate, asparagine or glutamine, can be incorporated into the peptide as the first amino acid, and then the remaining amino acids are coupled on. The allyl protective group can be removed by a two hour mixing of the peptide-resin with a solution of tetrakistriphenylphophine palladium (0) in a solution of chloroform containing 5% acetic acid and 2.5% N-methylmorpholine. The peptide resin can be washed three times with 0.5% N,N-diisopropylethylamine (DIEA) and 0.5% sodium diethyldithiocabamate in DMF. The amino terminal Fmoc group on the peptide chain can be removed by two incubations for 15 minutes each in 20% piperdine in DMF, and washed three times with DMF for 30 seconds each. The activator mix, N-methylmorpholine and HBTU in DMF, can be brought onto the column and allowed to couple the free amino terminal end to the carboxyl group generated by removal of the allyl group to cyclize the peptide. The peptide can cleaved from the resin as described in the general description of chemical peptide synthesis above and the

peptide purified by reverse phase-high pressure liquid chromatography (RP-HPLC). In a specific embodiment, in which the peptide to be cyclized does not contain an allyl protected amino acid, an allyl protected amino acid can be introduced into the sequence of the peptide, at the amino-terminus, carboxy-terminus or internally, such that the peptide can be cyclized.

β-hCG peptides can also be obtained by recombinant expression techniques. (See, *e.g.*, Sambrook et al., 1989, Molecular Cloning, A Laboratory Manual, Cold Spring Harbor Laboratory, 2d Ed., Cold Spring Harbor, New York, Glover, D.M. (ed.), 1985, DNA Cloning: A Practical Approach, MRL Press, Ltd., Oxford, U.K., Vol. I, II). The nucleic acid sequence encoding hCG has been cloned and the sequence determined (see Figure 4 and Xia, H., 1993, *J. Molecular Endocrinology* June 10; 1993:337-343; Sherman, G.B., 1992, *J. Molecular Endocrinology*, June 6, 1992:951-959; Gieseman, L.K. (ed.), 1991, *Basic and Chemical Endocrinology*, pp. 543-567; Ward et al., 1991, in *Reproduction in Domestic Animals*, 4th ed., P.T. Coppos, ed., pp. 25-80, Academic Press, New York) and can be isolated using well-known techniques in the art, such as screening a library, chemical synthesis, or polymerase chain reaction (PCR).

To recombinantly produce a β-hCG peptide, a nucleic acid sequence encoding the β-hCG peptide is operatively linked to a promoter such that the β-hCG peptide is produced from said sequence. For example, a vector can be introduced into a cell, within which cell the vector or a portion thereof is expressed, producing the β-hCG peptide. In a preferred embodiment, the nucleic acid is DNA if the source of RNA polymerase is DNA-directed RNA polymerase, but the nucleic acid may also be RNA if the source of polymerase is RNA-directed RNA polymerase or if reverse transcriptase is present in the cell or provided to produce DNA from the RNA. Such a vector can remain episomal or become chromosomally integrated, as long as it can be transcribed to produce the desired RNA. Such vectors can be constructed by recombinant

DNA technology methods standard in the art. Vectors can be plasmid, viral, or others known in the art, used for replication and expression in bacterial or mammalian cells. Expression of the sequence encoding the β-hCG peptide can be by any promoter known in the art to act in bacterial or mammalian cells. Such promoters can be inducible or constitutive. Such promoters include, but are not limited to: the SV40 early promoter region (Bernoist and Chambon, 1981, *Nature* 290:304-310), the promoter contained in the 3' long terminal repeat of Rous sarcoma virus (Yamamoto et al., 1980, *Cell* 22:787-797), the HSV-1 (herpes simplex virus-1) thymidine kinase promoter (Wagner et al., 1981, *Proc. Natl. Acad. Sci. USA* 78:1441-1445), the regulatory sequences of the metallothionein gene (Brinster et al., 1982, *Nature* 296:39-42), etc., as well as the following animal transcriptional control regions, which exhibit tissue specificity and have been utilized in transgenic animals: elastase I gene control region which is active in pancreatic acinar cells (Swift et al., 1984, *Cell* 38:639-646; Ornitz et al., 1986, *Cold Spring Harbor Symp. Quant. Biol.* 50:399-409; MacDonald, 1987, *Hepatology* 7:425-515); insulin gene control region which is active in pancreatic beta cells (Hanahan, 1985, *Nature* 315:115-122), immunoglobulin gene control region which is active in lymphoid cells (Grosschedl et al., 1984, *Cell* 38:647-658; Adames et al., 1985, *Nature* 318:533-538; Alexander et al., 1987, *Mol. Cell. Biol.* 7:1436-1444), mouse mammary tumor virus control region which is active in testicular, breast, lymphoid and mast cells (Leder et al., 1986, *Cell* 45:485-495), albumin gene control region which is active in liver (Pinkert et al., 1987, *Genes and Devel.* 1:268-276), alpha-fetoprotein gene control region which is active in liver (Krumlauf et al., 1985, *Mol. Cell. Biol.* 5:1639-1648; Hammer et al., 1987, *Science* 235:53-58), alpha 1-antitrypsin gene control region which is active in the liver (Kelsey et al., 1987, *Genes and Devel.* 1:161-171), beta-globin gene control region which is active in erythroid cells (Mogram et al., 1985, *Nature* 315:338-340; Kollias et

al., 1986, *Cell* 46, 89-94), myelin basic protein gene control region which is active in oligodendrocyte cells in the brain (Readhead et al., 1987, *Cell* 48:703-712), myosin light chain-2 gene control region which is active in skeletal muscle (Sani, 1985, *Nature* 314:283-286), and gonadotropin releasing hormone gene control region which is active in the hypothalamus (Mason et al., 1986, *Science* 234:1372-1378). The promoter element which is operatively linked to the nucleic acid encoding the β-hCG peptide can also be a bacteriophage promoter with the source of the bacteriophage RNA polymerase expressed from a gene for the RNA polymerase on a separate plasmid, e.g., under the control of an inducible promoter, for example, a nucleic acid encoding the β-hCG peptide operatively linked to the T7 RNA polymerase promoter with a separate plasmid encoding the T7 RNA polymerase.

In a less preferred embodiment, peptides can be obtained by proteolysis of hCG followed by purification using standard techniques such as chromatography (e.g., HPLC), electrophoresis, etc.

Also included within the scope of the invention are β-hCG peptide derivatives which are differentially modified during or after synthesis, e.g., by benzylation, glycosylation, acetylation, phosphorylation, amidation, pegylation, derivatization by known protecting/blocking groups, proteolytic cleavage, linkage to an antibody molecule or other cellular ligand, etc. In specific embodiments, the peptides are acetylated at the N-terminus and/or amidated at the C-terminus. Any of numerous chemical modifications may be carried out by known techniques, including but not limited to acetylation, formylation, oxidation, reduction; metabolic synthesis in the presence of tunicamycin; etc.

In another embodiment, the β-hCG peptide derivative is a chimeric, or fusion, protein comprising a functional β-hCG peptide joined at its amino- or carboxy-terminus via a peptide bond to an amino acid sequence of a different protein. In one embodiment, such a chimeric protein is

produced by recombinant expression of a nucleic acid encoding the protein (comprising a β-hCG-coding sequence joined in-frame to a coding sequence for a different protein). Such a chimeric product can be made by ligating the appropriate nucleic acid sequences encoding the desired amino acid sequences to each other by methods known in the art, in the proper coding frame, and expressing the chimeric product by methods commonly known in the art. Alternatively, such a chimeric product may be made by protein synthetic techniques, e.g., by use of a peptide synthesizer.

6.3.1. SOURCES OF hCG AND β-hCG

Native preparations (*i.e.* derived from naturally occurring sources and not recombinantly produced) of hCG and β-hCG can be obtained from a variety of sources. Both hCG and β-hCG are commercially available (*e.g.*, Sigma Chemical Company) and hCG is commercially available in a form suitable for therapeutic use in humans (*e.g.*, from Fujisawa, Wyeth-Ayerst Laboratories (APL™), Organon, Inc. (PREGNYL™) and Serono Laboratories, Inc. (PROFASI™)). hCG is also present at particularly high concentrations in the urine of women in the first trimester of pregnancy ("human early pregnancy urine"). Other sources include, but are not limited to, urine from women in the second and third trimesters of pregnancy, urine from patients with proteinuria, urine from patients having hCG secreting tumors or other cancer patients, and from pituitary glands.

Since the inventors have discovered that different sources of hCG have variable effects on HIV infection and cancer cell growth *in vitro* and *in vivo*, one aspect of the invention relates to assaying preparations of hCG for efficacy in treatment or prevention of HIV infection. The therapeutic effectiveness of hCG preparations and fractions can be tested by the *in vitro* or *in vivo* assays described in Section 6.4 *infra* or by any method known in the art. It is preferable to test the hCG preparation or fraction in an *in vitro* assay, *e.g.*, for HIV replication or transcription from

the HIV-1 LTR or *in vivo* in an animal model, such as HIV transgenic mice or SIV infected monkeys, before assaying the preparation in humans.

In a specific embodiment, a preparation comprising hCG is used that contains not only the hCG heterodimer but also peptide fragments thereof, *e.g.*, β chain peptides.

hCG and β-hCG can also be purified, or preferably partially purified, from any source known to contain hCG or β-hCG, *e.g.*, urine from pregnant women, using conventional techniques well-known in the art, such as affinity chromatography. For example, antibodies prepared against hCG or β-hCG can be used to prepare an affinity chromatography column which can be used to purify the proteins by well-known techniques (*see, e.g.*, Hudson & May, 1986, *Practical Immunology*, Blackwell Scientific Publications, Oxford, United Kingdom).

The β-hCG-related proteins are preferably prepared by any chemical or enzymatic synthesis method known in the art, as described *supra* in Section 6.1.2.

6.3.2. FRACTIONATION OF SOURCES OF hCG

The present inventors have found that the component(s) of a source of hCG having anti-HIV, anti-KS activity and/or pro-hematopoietic activities can be further isolated by fractionation of the source of hCG. The inventors have fractionated the active portions of the commercial hCG preparation APL™ (Wyeth-Ayerst) and human early (i.e. first trimester) pregnancy urine as described in Section 8 *infra*. Other sources of hCG include, but are not limited to, urine from women in the second and third trimester of pregnancy, urine from proteinuria patients (both pregnant women with preeclampsia and patients with nephrotic syndromes), urine from patients with hCG secreting tumors, and pituitary glands. However, those skilled in the art will appreciate that any source of hCG or β-hCG having anti-HIV and/or anti-KS activity and/or a pro-hematopoietic effect can be fractionated to further isolate the active components. The

source of hCG or β-hCG can be fractionated using any technique available in the art for the separation and isolation of molecules, for example but not limited to, sizing chromatography, ion-exchange chromatography, affinity chromatography, etc.

Additionally, the present inventors have found that different preparations of hCG and β-hCG have variable effects on HIV infection, KS cell growth and hematopoiesis both *in vitro* and *in vivo*. Specifically, the inventors found that among the commercial preparations of (non-recombinant) hCG they investigated, hCG from Fujisawa was the most effective, hCG APL™ (Wyeth-Ayerst) the next most effective, and PREGNYL™ (Organon) the next most effective in inhibiting HIV infection and replication. A highly purified hCG preparation and recombinant β-hCG were found not to be active in inhibiting HIV infection, KS cell growth and promoting hematopoiesis *in vitro*. In fact, the present inventors have shown that specific size fractions of an active hCG preparation (APL™; Wyeth Ayerst) and human early (*i.e.* first trimester) pregnancy urine have anti-HIV and pro-hematopoieitic activities in vitro and anti-KS activity both in vitro and in vivo, as described *infra* in Section 8.

These active fractions were eluted from the sizing matrix as or close to (i.e., within 5 fractions (where the fractions are 4 ml fractions using a SUPERDEX™ 200 (Pharmacia) column which is 26 mm^2 by 60 mm)) the fractions containing or that would contain material that is approximately 40 kD (±8 kD), 15 kD (±3 kD) and 2-3 kD (±2 kD) molecular weight. One skilled in the art would understand that these fractions could be subjected to further size fractionation to further isolate the component of these fractions having the anti-HIV and/or anti-KS activity. Additionally, other methods of fractionation, such as ion-exchange chromatography, affinity chromatography are well known in the art; those skilled in the art would be able to use any available fractionation techniques to obtain the active fractions from the active hCG preparations and human

early (first trimester) pregnancy urine (or any other preparation containing hCG or β-hCG).

In a specific embodiment, the invention provides a first composition comprising one or more first components of a second composition comprising native hCG or native β-hCG, said first components being separated from other components of the hCG or β-hCG sample, said first components being active to inhibit HIV infection or replication or Kaposi's sarcoma or having a pro-hematopoietic activity, and said second composition being active to inhibit HIV infection or replication or Kaposi's sarcoma or having a pro-hematopoietic activity, and said native hCG or native β-hCG not being purified to homogeneity in said second composition.

In particular the invention provides a composition comprising components which have been separated from other components of the native hCG or native β-hCG sample by sizing column chromatography, preferably where the components elute from a gel filtration, preferably a SUPERDEX™ 200, sizing column with an apparent approximate molecular weight of 40 kD, 14 kD or 2-3 kD as determined relative to the elution of a hCG heterodimer, having a molecular weight of 77 kD, and a β-hCG core protein (β-hCG amino acids 6-40 linked via a disulfide bond to β-hCG amino acids 55-92, as depicted in Figure 4 (SEQ ID NO:2)), having a molecular weight of 10 kD. hCG preparations and fractions of hCG preparations can be screened for efficacy in inducing proliferation of hematopoietic cells by the assays described in Sections 6.4, 7 and 8 *infra* or by any method known in the art.

Thus, in a preferred embodiment, a fraction, particularly a size fraction, of a source of native hCG or native β-hCG active in promoting hematopoiesis, particularly a size fraction of approximately 40 kD, 15 kD or 2-3 kD, is used to treat or prevent a hematopoietic deficiency.

The inventors have fractionated the active portions of the commercial hCG preparation APL™ (Wyeth-Ayerst) and human early (*i.e.* first trimester) pregnancy urine as described in Section 8 *infra*. Other sources of hCG include, but are not

limited to, urine from women in the second and third trimester of pregnancy, urine from proteinuria patients (both pregnant women with preeclampsia and patients with nephrotic syndromes), urine from patients with hCG secreting tumors, and pituitary glands. However, those skilled in the art will appreciate that any source of hCG or β-hCG having anti-HIV activity and/or anti-KS activity and/or a pro-hematopoietic effect can be fractionated to further isolate the active components. The source of hCG or β-hCG can be fractionated using any technique available in the art for the separation and isolation of molecules, for example but not limited to, sizing chromatography, ion-exchange chromatography, affinity chromatography, etc.

Briefly, by way of example but not by way of limitation, urine can be prepared for fractionation as follows:

Urine is collected and stored either frozen or refrigerated for not more than two (2) days. Then, sodium azide is then added at a concentration of 1 gram/liter and the sample is stored frozen until sufficient material is collected for the fractionation.

At this point, the urine is thawed over night, the pH adjusted to 7.2 to 7.4 with sodium hydroxide and then centrifuged to remove any precipitate (alternatively, the precipitate can be allowed to sediment, *e.g.*, for 1 hour at room temperature, approximately 75% of the supernatant is decanted, the remainder of the supernatant and the precipitate is centrifuged to pellet the precipitate, and the supernatant decanted and added to the first volume of decanted supernatant). The urine is then filtered through, *e.g.*, a 45 micron filter to remove any remaining particulate matter.

Next, the urine is concentrated using any concentration method available in the art which does not remove higher molecular weight material, *e.g.*, material larger than 3,000 daltons in molecular weight; for example, the material may be concentrated using a Pellicon (Millipore) filtration system with a membrane

filter cassette having a molecular weight cut off of 3,000 daltons. Concentration with the Pellicon filtration system using the 3,000 molecular weight membrane filter cut off concentrates 30 liters of urine to 500 ml (i.e., a 60-fold concentration) overnight.

To remove salts and lipids, the concentrate can then be passed over a column containing a large volume of Sephadex G25 resin in 0.05 M ammonium bicarbonate (for example, 250 ml of the concentrate can be passed over a column of approximately 1.7 liters, washing the column with 25% ethanol between runs to remove adsorbed lipids and glycoprotein). The resulting desalted and delipidated urine concentrate is then lyophilized.

The lyophilized urine material or commercial hCG preparation (or any source of native hCG or native β-hCG) is resuspended in either phosphate buffered saline (PBS-- 30 mM sodium phosphate buffer, pH 8.3) or in 0.10 M ammonium bicarbonate at a concentration and in a volume appropriate for the column upon which the sample will be loaded, for example, but not limited to 0.5 grams of protein in 6 ml (i.e., approximately 83 mg/ml). It is within the skill of the skilled artisan to determine the concentration and volume of the sample to be subjected to fractionation.

The sample can then be fractionated by any method known in the art for the separation of proteins. A preferred method is high resolution gel filtration on a Pharmacia pre-packed SUPERDEX™ 200 column (26/60) by HPLC using any available HPLC apparatus, *e.g.*, with a Hewlett Packard 1050 HPLC equipped with a photodiode array detector. The resuspended sample is loaded onto the column in 30 mM phosphate buffer, pH 8.3, and the material can then be eluted from the column with 30 mM sodium phosphate buffer, pH 7.0; 2M NaCl. Fractionation can also be performed using other types of chromatography matrices e.g., heparin, DEAE-cellulose, Sephadex A50, Sephadex G100, phenyl sepharose, or any sizing, ion-exchange, affinity chromatography or any other chromatography matrix available in the art. The column

chromatography can also be run using any method available in the art, e.g., standard gravity flow or low pressure chromatography, FPLC, or reverse phase HPLC.

Many separation techniques are known in the art. Those skilled in the art would understand how to apply these known techniques to the fractionation of hCG preparations.

Once the material has been fractionated, any method known in the art, such as but not limited to, those described in sections 6.4, 7, and 8 *infra*, can be used to determine which fractions have anti-HIV activity and/or anti-KS activity and/or a pro-hematopoietic effect.

When fractionating by size, such as fractionation on the SUPERDEX™ 200 column, the apparent molecular weight of material in the fractions can be determined by the relative elution of those fractions compared with the elution of specific hCG and β-hCG species having a known molecular weight or with the elution of known protein size markers. In general, proteins elute from a sizing column as a function of their molecular weight. The elution of, for example, hCG and the β-hCG core protein can be determined by assaying the column chromatography fractions for the presence of hCG and the β-hCG core protein, or any hCG or β-hCG species, by any immunoassay technique available in the art, such as radioimmunoassays (either liquid or solid phase), enzyme-linked assays or ELISA assays.

Antibodies, either polyclonal or, preferably, monoclonal, can be generated against hCG or the β-hCG core protein by any method known in the art. Preparation of monoclonal antibodies against hCG and β-hCG species have been described in the art, *see, e.g.*, O'Connor et al., 1994, *Endocrine Reviews* 15:650-683; Krichevsky et al, 1991, *Endocrinology* 128:1255-1264; and Krichevsky et al., 1988, *Endorcrinology* 123:584-593. For the production of antibodies, various host animals can be immunized by injection with hCG, the β-hCG core protein or any other species of hCG, including but not limited to rabbits, mice, rats, etc. Various adjuvants may be used to increase the

immunological response, depending on the host species, and including but not limited to Freund's (complete and incomplete), mineral gels such as aluminum hydroxide, surface active substances such as lysolecithin, pluronic polyols, polyanions, peptides, oil emulsions, keyhole limpet hemocyanins, dinitrophenol, and potentially useful human adjuvants such as BCG (bacille Calmette-Guerin) and corynebacterium parvum. For preparation of monoclonal antibodies, any technique which provides for the production of antibody molecules by continuous cell lines in culture may be used. For example, the hybridoma technique originally developed by Kohler and Milstein (1975, Nature 256:495-497), as well as the trioma technique, the human B-cell hybridoma technique (Kozbor et al., 1983, Immunology Today 4:72), and the EBV-hybridoma technique to produce human monoclonal antibodies (Cole et al., 1985, in Monoclonal Antibodies and Cancer Therapy, Alan R. Liss, Inc., pp. 77-96). Monoclonal cells lines can then be screened for binding to the particular hCG or β-hCG species using the purified species in any type of immunoassay available in the art (*see, e.g.*, Erlich et al., 1985, *Am. J. Reprod Immunol. Microbiol.* 8:48).

The fractions can then be assayed for the presence of the hCG or β-hCG species using a monoclonal antibody specific for the hCG or β-hCG species. The assay can be performed by any method known in the art. For example, an immunoradiometric assay (IRMA) can be used (Krichevsky et al., 1988, *Endocrinology* 123:584-593). Briefly, the IRMA assay is performed by adsorbing an antibody against the hCG or β-hCG species onto the surface of wells of a microtiter plate by incubation in a coating buffer (0.2 M sodium bicarbonate, pH 9.5) overnight at 4°C. The residual non-specific binding sites are blocked by the addition of a 1% bovine serum albumin solution (with 0.1% sodium azide) to the wells for 3 hours at room temperature, and the wells of the microtiter plate are then washed with deionized water. An aliquot of the fraction in assay buffer (0.01 M sodium phosphate, 0.15 M NaCl, 0.01 M EDTA, 0.1% sodium azide, 0.1%

bovine γ-globulin, pH 7.4) is incubated in the wells for 24 hours at room temperature. The sample is then removed and the wells washed with deionized water. A solution of a second antibody specific for the hCG or β-hCG species, which antibody has been iodinated with I^{125}, (approximately 40,000 cpm/well) is incubated in the wells for 24 hours at room temperature. The iodinated antibody solution is removed and the wells washed five times with deionized water. The level of radioactivity in each well is then determined in a scintillation counter which can measure γ-irradiation.

6.4. ASSAYS FOR INDUCTION OF HEMATOPOIETIC CELL PROLIFERATION

The Therapeutics of the invention are preferably tested *in vitro*, and then *in vivo* for the desired therapeutic or prophylactic activity, prior to use in humans. Any *in vitro* or *in vivo* assay known in the art to measure a pro-hematopoietic effect, *i.e.* the ability to induce hematopoietic cell proliferation *in vitro* or production of one or more hematopoietic cell types *in vivo*, such as those described in Section 7 *infra*, can be used to test the efficacy of a Therapeutic of the invention.

A specific embodiment provides a method for screening a preparation comprising hCG or an hCG α chain or hCG β chain or a derivative of hCG or of said alpha or beta chain or a fraction of a source of native hCG or native β-hCG, for pro-hematopoietic activity comprising assaying said preparation for the ability to induce an increase in hematopoietic cell numbers. In one embodiment, the preparation is screened by a method comprising measuring the number of colonies formed from hematopoietic stem or progenitor cells, which cells have been contacted with the preparation; and comparing the number of colonies formed from the cells contacted with the preparation with the number of colonies formed from cells not so contacted with the preparation, wherein a higher number of colonies formed from said contacted cells indicates that the preparation has pro-hematopoietic activity. In another

embodiment, the preparation is screened by a method comprising measuring the number of $CD4^+$ T cells in an SIV infected monkey, which monkey has been exposed to the preparation; and comparing the number of $CD4^+$ T cells in the monkey which has been exposed to the preparation with the number of $CD4^+$ T cells in a monkey not so exposed, wherein a higher number of $CD4^+$ T cells in said exposed monkey indicates that the preparation has pro-hematopoietic activity.

Specifically, to assay a Therapeutic *in vitro*, one could examine the effect of the Therapeutic on proliferation of hematopoietic cells *in vitro*. For example, to assay colony-forming units (a progenitor cell), briefly, the hematopoietic cells are cultured for an appropriate amount of time, such as 5 to 20 days and preferably 10 days, in the presence of (or otherwise exposed to) the Therapeutic to be tested, and then colony assays are performed to determine the number of colonies formed in comparison to the number of colonies formed by cells cultured in the absence of the Therapeutic. For example, hematopoietic progenitor cells can be isolated from bone marrow or cord blood, seeded in methylcellulose in the presence of absence of the Therapeutic, and then colony numbers determined after 10 days of culture. An increase in colony numbers in cells contacted with the Therapeutic indicates that the Therapeutic has activity in inducing proliferation of hematopoietic cells. Thus, for example, depending on the progenitor cell desired to be assayed, CFU-GM, CFU-GEMM, etc., assays can be done.

Therapeutics can also be tested *in vivo* for activity in increasing the numbers of hematopoietic cells. Preferably, Therapeutics are tested in animal models of hematopoietic disorders before testing them in human patients. For example, but not by way of limitation, a Therapeutic can be tested in rhesus monkeys infected with SIV, particularly SIV_{mac251} which induces a syndrome in monkeys similar to human AIDS (Kestler, H. et al., 1990, *Science* 248:1109-1112), and which are deficient in $CD4^+$ T cells. The Therapeutic to be tested can be administered to the infected monkeys; then the

blood or bone marrow of the infected monkeys can be examined for an increase in $CD4^+$ T cells or any other hematopoietic cell type for which the monkey is deficient. An increase in numbers of the hematopoietic cell demonstrates that the Therapeutic is useful for treating diseases and disorders associated with hematopoietic deficiencies. Any animal model of an anemia can be similarly used for testing.

Therapeutics can be tested in human patients, preferably after tests *in vitro* and/or *in vivo* in an animal model, with hematopoietic deficiencies, for example but not limited to, deficiencies associated with HIV infection such as anemia, neutropenia, thrombocytopenia, or $CD4^+$ T cell lymphocyte deficiency, for activity in increasing numbers of hematopoietic cells for which the patient is deficient. Briefly, the Therapeutic is administered, for example by intramuscular injection two to three times per week, to the patient suffering from the hematopoietic deficiency. The subject's blood or bone marrow is assayed before and after treatment with the Therapeutic for an increase in the hematopoietic cell numbers. Therapeutics which cause an increase in hematopoietic cell numbers are useful for treatment of diseases and disorders associated with hematopoietic deficiencies.

Assays for hematopoietic cell proliferation in the blood or bone marrow can be accomplished by any method well known in the art. For example, blood can be drawn and blood cell numbers can be determined by routine clinical laboratory tests for red blood cells, platelets, neutrophils, lymphocytes, etc. Additionally, colony assays on isolated bone marrow can be performed to assess increases in stem or progenitor cells. For example, bone marrow can be sampled and bone marrow cells evaluated for stem and progenitor cell colony formation. Briefly, cells are seeded in methylcellulose, cultured for 12 to 14 days, and then scored for colony formation where aggregates containing more than 50 cells are counted as a colony (*see, e.g.*, Lunardi-Iskandar, Y. et al., 1995, *Nature* 375:64-68; Louache, R. et al., 1992,

Blood 180:2991-2999; Lunardi-Iskandar, Y. et al., 1989, *J. Clin. Invest.* 83:610-615). Bone marrow progenitors which can be evaluated by this colony assay include, but are not limited to, CFU-Mix, BFU-e and CFU-GM. As an alternative to colony assays for detection and quantitation of stem and/or progenitor cells, immunological detection methods can be employed, based on the antigens expressed by the particular cell type (*see, e.g.*, the relevant discussion in Section 6.2 hereinabove).

6.5. THERAPEUTIC COMPOSITIONS AND METHODS OF ADMINISTRATION

The invention provides methods of treatment and prevention by administration to a subject of an effective amount of a Therapeutic of the invention. The subject is preferably an animal, including, but not limited to, animals such as cows, pigs, horses, chickens, cats, dogs, etc., and is preferably a mammal, and most preferably human. In a specific embodiment, the subject is a human not afflicted with a cancer which secretes hCG or hCG fragments and, more particularly, not afflicted with Kaposi's Sarcoma.

One embodiment of the invention provides for methods of administering a pharmaceutical composition which is comprised of a therapeutically effective amount of hematopoietic cells, the production of which has been increased by contact with a Therapeutic of the invention. In a particular embodiment, the pharmaceutical composition contains hematopoietic cells made recombinant by gene therapy methods. These hematopoietic cells can be provided to a patient by any method known in the art, preferably by intravenous delivery. Various delivery systems are known and can be used to administer a Therapeutic of the invention, *e.g.*, encapsulation in liposomes, microparticles, microcapsules, recombinant cells capable of expressing the Therapeutic, receptor-mediated endocytosis (see, *e.g.*, Wu and Wu, 1987, J. Biol. Chem. 262:4429-4432), construction of a Therapeutic nucleic acid as part of a retroviral or other vector, etc. Methods of introduction include but are not limited to

intradermal, intramuscular, intraperitoneal, intravenous, subcutaneous, intranasal, epidural, and oral routes. The compounds may be administered by any convenient route, for example by infusion or bolus injection, by absorption through epithelial or mucocutaneous linings (*e.g.*, oral mucosa, rectal and intestinal mucosa, etc.) and may be administered together with other biologically active agents.

Administration can be systemic or local. In addition, it may be desirable to introduce the pharmaceutical compositions of the invention into the central nervous system by any suitable route, including intraventricular and intrathecal injection; intraventricular injection may be facilitated by an intraventricular catheter, for example, attached to a reservoir, such as an Ommaya reservoir. Pulmonary administration can also be employed, *e.g.*, by use of an inhaler or nebulizer, and formulation with an aerosolizing agent. It may be desirable to administer the pharmaceutical compositions of the invention locally to the area in need of treatment; this may be achieved, for example and not by way of limitation, by topical application, by injection, by means of a catheter, by means of a suppository, or by means of an implant, said implant being of a porous, non-porous, or gelatinous material, including membranes, such as sialastic membranes, or fibers.

In one embodiment, the Therapeutic can be delivered in a vesicle, in particular a liposome (see Langer, *Science* 249:1527-1533 (1990); Treat et al., in *Liposomes in the Therapy of Infectious Disease and Cancer*, Lopez-Berestein and Fidler (eds.), Liss, New York, pp. 353-365 (1989); Lopez-Berestein, *ibid.*, pp. 317-327; see generally *ibid.*)

In another embodiment, the Therapeutic can be delivered in a controlled release system. In one embodiment, a pump may be used (see Langer, *supra*; Sefton, *CRC Crit. Ref. Biomed. Eng.* 14:201 (1987); Buchwald et al., *Surgery* 88:507 (1980); Saudek et al., *N. Engl. J. Med.* 321:574 (1989)). In another embodiment, polymeric materials can be used (see Medical Applications of Controlled Release, Langer and Wise

(eds.), CRC Pres., Boca Raton, Florida (1974); Controlled Drug Bioavailability, Drug Product Design and Performance, Smolen and Ball (eds.), Wiley, New York (1984); Ranger and Peppas, *J. Macromol. Sci. Rev. Macromol. Chem.* 23:61 (1983); see *also* Levy et al., *Science* 228:190 (1985); During et al., *Ann. Neurol.* 25:351 (1989); Howard et al., *J. Neurosurg.* 71:105 (1989)). In yet another embodiment, a controlled release system can be placed in proximity of the therapeutic target, i.e., the brain, thus requiring only a fraction of the systemic dose (see, e.g., Goodson, in Medical Applications of Controlled Release, *supra*, vol. 2, pp. 115-138 (1984)).

Other controlled release systems are discussed in the review by Langer (*Science* 249:1527-1533 (1990)).

In a specific embodiment where the Therapeutic is a nucleic acid encoding a protein Therapeutic, the nucleic acid can be administered by gene therapy methods as described *supra* Section 6.1.4.

The present invention also provides pharmaceutical compositions. Such compositions comprise an amount of a Therapeutic effective to increase hematopoietic cell production, and a pharmaceutically acceptable carrier. In a specific embodiment, the term "pharmaceutically acceptable" means approved by a regulatory agency of the Federal or a state government or listed in the U.S. Pharmacopeia or other generally recognized pharmacopeia for use in animals, and more particularly in humans. The term "carrier" refers to a diluent, adjuvant, excipient, or vehicle with which the Therapeutic is administered. Such pharmaceutical carriers can be sterile liquids, such as water and oils, including those of petroleum, animal, vegetable or synthetic origin, such as peanut oil, soybean oil, mineral oil, sesame oil and the like. Water is a preferred carrier when the pharmaceutical composition is administered intravenously. Saline solutions and aqueous dextrose and glycerol solutions can also be employed as liquid carriers, particularly for injectable solutions. Suitable pharmaceutical excipients

include starch, glucose, lactose, sucrose, gelatin, malt, rice, flour, chalk, silica gel, sodium stearate, glycerol monostearate, talc, sodium chloride, dried skim milk, glycerol, propylene glycol, water, ethanol and the like. The composition, if desired, can also contain minor amounts of wetting or emulsifying agents, or pH buffering agents. These compositions can take the form of solutions, suspensions, emulsion, tablets, pills, capsules, powders, sustained-release formulations and the like. The composition can be formulated as a suppository, with traditional binders and carriers such as triglycerides. Oral formulation can include standard carriers such as pharmaceutical grades of mannitol, lactose, starch, magnesium stearate, sodium saccharine, cellulose, magnesium carbonate, etc. Examples of suitable pharmaceutical carriers are described in "Remington's Pharmaceutical Sciences" by E.W. Martin. Such compositions will contain a therapeutically effective amount of the Therapeutic, preferably in purified form, together with a suitable amount of carrier so as to provide the form for proper administration to the patient. The formulation should suit the mode of administration.

In a preferred embodiment, the composition is formulated in accordance with routine procedures as a pharmaceutical composition adapted for intravenous administration to human beings. Typically, compositions for intravenous administration are solutions in sterile isotonic aqueous buffer. Where necessary, the composition may also include a solubilizing agent and a local anesthetic such as lignocaine to ease pain at the site of the injection. Generally, the ingredients are supplied either separately or mixed together in unit dosage form, for example, as a dry lyophilized powder or water free concentrate in a hermetically sealed container such as an ampoule or sachette indicating the quantity of active agent. Where the composition is to be administered by infusion, it can be dispensed with an infusion bottle containing sterile pharmaceutical grade water or saline. Where the composition is administered by injection, an

ampoule of sterile water for injection or saline can be provided so that the ingredients may be mixed prior to administration.

The Therapeutics of the invention can be formulated as neutral or salt forms. Pharmaceutically acceptable salts include those formed with free amino groups such as those derived from hydrochloric, phosphoric, acetic, oxalic, tartaric acids, etc., and those formed with free carboxyl groups such as those derived from sodium, potassium, ammonium, calcium, ferric hydroxides, isopropylamine, triethylamine, 2-ethylamino ethanol, histidine, procaine, etc.

The amount of the Therapeutic of the invention or titer of hematopoietic cells or recombinant hematopoietic cells which will be effective in the treatment of a particular disorder or condition will depend on the nature of the disorder or condition, and can be determined by standard clinical techniques. In addition, *in vivo* and/or *in vitro* assays may optionally be employed to help identify optimal dosage ranges. The precise dose to be employed in the formulation will also depend on the route of administration, and the seriousness of the disease or disorder, and should be decided according to the judgment of the practitioner and each patient's circumstances. For example, therapy of at least 15,000 I.U. and up to 45,000 I.U. hCG weekly was shown to be effective and well tolerated in humans. Weekly doses of 6,000 I.U. in monkeys and 300-500 I.U. in mice were also shown to be effective. Additionally, predicted suitable doses of a β-hCG peptide for treatment or prevention of diseases and disorders in which increased numbers of one or more hematopoietic cell types are desirable include, but are not limited to, 1 to 1000 micrograms per week. Routes of administration of a Therapeutic include, but are not limited to, subcutaneously, intramuscularly or intravenously. Effective doses may be extrapolated from dose-response curves derived from *in vitro* or animal model test systems.

Suppositories generally contain active ingredient in the range of 0.5% to 10% by weight; oral formulations preferably contain 10% to 95% active ingredient.

The invention also provides a pharmaceutical pack or kit comprising one or more containers filled with one or more of the ingredients of the pharmaceutical compositions of the invention. Optionally associated with such container(s) can be a notice in the form prescribed by a governmental agency regulating the manufacture, use or sale of pharmaceuticals or biological products, which notice reflects approval by the agency of manufacture, use or sale for human administration.

7. EXAMPLE: EFFECTS OF PREPARATIONS OF hCG, β-hCG and β-hCG PEPTIDES ON HEMATOPOIESIS

Human Chorionic Gonadotropin (hCG), a glycoprotein hormone produced in early pregnancy, consists of two sub-units, α and β, which associate non covalently to form a heterodimer which embodies its hormonal activity. Some partially purified preparations of hCG and β-hCG and some β-chain fragments stimulate the growth of hematopoietic progenitors, for example β-hCG peptides having amino acid sequences of amino acid numbers 45-57 (SEQ ID NO:6), 109-119 (SEQ ID NO:7), circularized 44-57, where cysteine is substituted for the amino acid at position 44 (SEQ ID NO:26), and peptides of amino acid numbers 45-57 (SEQ ID NO:6) linked at the C-terminus via a peptide bond to the N-terminus of amino acids 109-119 (SEQ ID NO:7) or linked at the N-terminus via a peptide bond to the C-terminus of amino acids 110-119 (SEQ ID NO:27); or a peptide of amino acids 47-57 (SEQ ID NO:28) linked at the C-terminus via a peptide bond to the N-terminus of amino acids 108-119 (SEQ ID NO:29) of the β-hCG sequence depicted in Figure 4 (portions of SEQ ID NO:2). The peptides having an amino acid sequence of amino acid numbers 7-45, 47-55, 46-65, and 48-56 (SEQ ID NOS:21 and 33-35, respectively) of β-hCG (Figure 4 (SEQ ID NO:2) also exhibit activity in *in vitro* assays.

Furthermore, in 3 of 3 SIV acutely infected rhesus macaque monkeys the same preparation of hCG (pre-screened for anti viral activity) at a dose of 6,000 IU per week, led to a reduction of SIV in plasma, an increase in CD4· T cells and weight gain. Examples of pro-hematopoietic effects are observed in preliminary studies of a limited number of patients treated with some commercial hCG products. Factors such as patient stage, total weekly dose, and manufacturer source very likely play a role in the variability of response.

7.1. EFFECTS OF hCG PREPARATIONS IN SIV INFECTED RHESUS MONKEYS

Events early in HIV infection are thought to be critical to subsequent AIDS pathogenesis. Although the early events in HIV infection are difficult to study in humans, they can be readily investigated in the SIV infected rhesus monkey animal model (Letvin et al., 1990, *J. AIDS* 3:1023-1040). SIV and HIV-1 are similar in many of their biological and physical properties including their genomic structure. In addition, SIV_{mac251}, unlike several other SIV isolates, induces a syndrome in experimentally infected rhesus macaques that is similar to human AIDS (Kestler et al., 1990, *Science* 248:1109-1112).

The effect of the same commercially available hCG preparation (APL™, Wyeth Ayerst), which had been prescreened for anti-viral and anti-KS activity, was studied in five adult male rhesus monkeys who were intravenously inoculated with cell free SIV_{mac251} ($10^{4.5}$ $TCID_{50}$/ml). In all animals, SIV p27 was apparent in plasma 14 days after infection, reaching a maximum by about day 20 (not shown). Treatment with systemic injections (3,000 IU, 2 times weekly) of the active commercial preparation of hCG (APL™), was initiated 3 weeks after SIV inoculation. Two months post-inoculation, the characteristic increase of SIV p27 antigen (Figure 1A), reduction of CD4· T cells (Figure 1B), and weight loss (Figure 1C) occurred in 2 of 2 untreated infected monkeys. In

contrast, the 3 infected monkeys treated with this hCG preparation showed weight gain characteristic of uninfected animals of this age (Figure 1C), a marked decrease in SIV p27 (Figure 1A) and an increase in CD4+ T cells to normal levels (Figure 1B). These effects were maintained over the 7 months the animals were followed. These results show that this commercially available hCG preparation can control SIV_{mac251} acute infection, increase CD4+ T cells, and promote weight gain in SIV infected rhesus monkeys and that these effects can be maintained. The animals were followed for 7 months, and no evidence of disease or SIV resistance to the hCG preparation developed.

In Figure 1D, results are shown from 4 uninfected controls: 2 received the hCG preparation and 2 received the diluent without the hCG. There is a slight increase in the CD4+ T cells in the treated animals (increasing from 460 mm^3 to 520 mm^3 and from 470 mm^3 to 650 mm^3) (Figure 1D). The 2 treated animals also showed a 1 to 2 kg weight gain (not shown).

7.2. EARLY STUDIES OF SOME hCG PREPARATIONS IN PATIENTS WITH HIV-1 DISEASE

The incidence of KS is greatly increased in HIV-infected persons (Friedman-Kien et al., 1981, *J. Am. Acad. Dermatol.* 5:468-473). Based on experimental studies of the killing effect of some hCG preparations on KS Y-1 cells, clinical trials with some commercially available preparations of hCG given either intralesionally (Hermans et al., 1995, *Cellular and Molecular Biology* 3:357-364; Gill et al., 1996, *New Engl. J. Med.* 335:1261-1269, Harris, P.J., 1995, *The Lancet* 346:118-119) or systemically to KS patients have shown that cutaneous KS lesions were reduced via cell killing by apoptosis following intralesional inoculation (Lunardi-Iskandar et al., 1995, *Nature* 375:64-68; Hermans et al., 1995, *Cellular and Molecular Biology* 3:357-364; Gill et al., 1996, *New Engl. J. Med* 335: 1261-1269) and induced

regression of advanced KS disease treated by systemic delivery.

Clinical trials reported herein were undertaken in Belgium and California to evaluate the anti-KS properties of systemic hCG therapy with or with out concomitant intralesional therapy. Use of anti-viral protease and non-protease inhibitors was not restricted. A total of 47 patients were enrolled under protocols of compassionate use sanctioned by the Institutional Review Boards of the respective centers. 29 patients were treated in Belgium, either on a protocol to investigate intralesional and systemic treatment of cutaneous KS (n=15), or in the pre-clinical phase of that protocol (n=4), or on compassionate use for systemic KS or HIV infection (n=10). The protocol involved intralesional administration of 500 IU hCG (PREGNYL™) to 4 lesions for 2 weeks, followed by subcutaneous administration of 2,500 IU hCG (PREGNYL™) 5 days per week for 4 to 6 weeks. Additional systemic intramuscular or subcutaneous hCG treatment with either PREGNYL™, APL™, or STERIS™ (one patient) was provided as ongoing therapy in some patients or as part of compassionate use protocols.

A total of 18 patients were treated in California with at least 1 month of follow-up as part of an ongoing protocol to evaluate systemic hCG therapy for cutaneous KS. These patients received either 5000 IU of APL™ subcutaneously 7 days per week, 10,000 IU subcutaneously 3 times per week, or 10,000 IU subcutaneously 7 days per week.

Overall 30 patients were on pre-existing, anti-viral therapy (19 on RT inhibitors and 11 on protease inhibitors), 11 were on no anti-virals and 8 were missing information. One patient, PH-RF, was on 3TC therapy before hCG therapy, and despite poor compliance, had an hCG response for visceral KS and viral load, which declined to undetectable on hCG alone.

Thirty-six patients survived the study, 7 (PH-LFA, PH-DD, PH-PJ, PO-BO, PO-RB, PH-JJ, PH-MH) died either from opportunistic infections or multiple organ failure. The

vital status of 1 patient is unknown. Two patients, PH-DD and PH-OJ, discontinued hCG treatment because of cholestasis. PH-DD was on concomitant anti-mycobacterial therapy which was felt to be a contributing factor. PH-OJ had preexisting cholestasis, which was exacerbated by the hCG treatment with a marked increase in alkaline phosphatase and rise in bilirubin which required hospitalization (PH). These values declined by 2-fold following discontinuation of hCG therapy. These cases raise the possibility that liver toxicity may be a rare complication of hCG therapy.

Early clinical experience with relatively low dose intralesional hCG administration for KS indicated partial or complete regression of treated lesions, including 3 of the first 4 patients in the initial pilot study in Belgium (Hermans et al.,1995, *Cellular and Molecular Biology* 3:357-364) as well as a dose dependent effect between 16% (250 IU) and 83% (2,000 IU) in patients reported from California (Gill et al., 1996, *New Engl. J. Med.* 335:1261-1269), and other cases showing striking clearance of visceral (lung and gastrointestinal) KS in very advanced disease following systemic therapy with hCG APL™ or PREGNYL™ within 1 to 3 months of initiating therapy.

Among the 30 cases with cutaneous Kaposi's Sarcoma, 12 were treated with intralesional followed by systemic therapy in Belgium and 18 with systemic therapy only in California. Complete (2/12, Belgium; 2/18, California) and partial (5/12, Belgium; 4/18, California) responses were observed while progressive disease was noted among 2/12 from Belgium and 10/18 in California. The overall response rate for the study (CR + PR) was 43% (13/30). The response rate in the group administered hCG both intralesionally plus systemicly group was 58%, while the response rate was 33% in the group receiving only the systemic treatment. Among 8 patients with both visceral and cutaneous KS treated in Belgium with very advanced pulmonary or gastric lesions, 3 patients experienced complete remissions, 2 patients exhibited tumor stabilization

and 3 progressed, in each case after failure of conventional cytotoxic therapy.

AIDS patients treated with hCG therapy were tested for increases in $CD4^+$ T cell levels (in numbers of cells per mm^3) and decrease in viral load by one of the following assays for determining viral load: NASBA (Louache, et al., 1992, *Blood* 180:2991-2999; Geller, et al., 1985, *Archs. Path. Lab. Met.* 109:138-145), which has a lower detection limit of 4,000 copies; Roche Amplicor, with a lower detection limit of 200 copies; RT-PCR, with a lower detection limit of 100 copies; or TCID assay in which the infection of PBMCs in co-culture is determined (Popovic et al., 1984, *Science* 204:497-500). As viral load was assayed retrospectively, the viral load results were not a factor in guiding choice of therapy or changes in therapy. Each patient served as their own control and change in viral load (0.7 log change between baseline and subsequent post hCG viral load, scored as significant) was the endpoint measurement for this analysis. For analysis of the anti-viral effect, in addition to the 10 patients undergoing with synchronous hCG and other anti-viral therapy, 6 patients were excluded because of a lack of base line viral load or insufficient follow up before hCG therapy was stopped or additional anti-viral therapy was started.

Among the 16 cases, 1 (PH-OJ) experienced a fall in viral load of 0.7 log on 2 successive tests at least 1 month apart while on stable anti-viral therapy (see Figures 2A and B), 11 were non responding and 2 (PH-VE and PHGRX) manifested an increase in viral load of at least 0.7 log after hCG therapy on 2 successive tests at least 1 month apart. As illustrated in Figures 2C and D, another patient (PG-1), initially on hCG alone and classified as non responsive by study criteria (2 consecutive values of 0.7 log decrease in viral load over 1 month) on hCG alone, experienced a steady decline in viral load but the second qualifying >0.7 log viral load drop was measured 2 weeks after non protease inhibitor therapy was begun. Because of this short window, it is likely that this second stable viral load point is

accounted for by hCG rather than the newly introduced anti-virals. It is noteworthy that CD4+ T cell levels were not significantly altered in this case but, the patient's KS progressed, documenting a dissociation of various hCG effects.

Among the 6 cases being treated with hCG alone (i.e. without other anti-viral therapies) with analyzable data, all were scored as non responsive to the hCG therapy by the scoring criteria although one case (PG-1) noted above (and illustrated in Figures 2C and D) is a probable responder. An additional patient on hCG alone (PG-8; Figures 2E and F) experienced a sustained fall in viral load of 0.5 log over a 2.8 month period of treatment on hCG alone until KS lesions progressed, at which time hCG therapy was discontinued. Thus of the 7 analyzable patients on hCG alone, 4 exhibited a downward trend in viral load, 2 patients showed an increase in viral load, and 1 patient was stable.

To more fully evaluate all data from patients on hCG alone or with stable antiviral therapy, all eligible data points were plotted, as shown in Figure 8A, indicating the coordinates for each data point pre and post therapy, with values on the line representing no change in viral load. Values are distributed more or less equally above and below the line with no obvious trend to suggest a strong anti viral effect. To evaluate a dose response relationship between hCG and viral load, regression analysis for patients on hCG, alone or with stable antiviral therapy is shown in Figure 8C. There was no detectable effect of higher hCG dose on viral load level ($r=-.089$, $p=0.285$, $N=147$ measurements). An analysis by different CD4 strata did not show any significant trends to suggest that level of immunity impacted the hCG effect.

Among the 22 patients with analyzable CD4+ T cell data, 5 demonstrated a pro-CD4+ T cell effect (PH-VE, PH-RF, PG-9, PG-17, and PG-19) characterized by a 50% rise in CD4+ T cell count sustained over at least a one month period, as demonstrated by plotting the data from at least two patients

(PH-VE--Figures 2G and H and PG-17--Figures 2I and J). Of these 5 patients, concomitant stable non protease anti-virals were administered to 2 patients, stable protease inhibitors in 2 cases and hCG preparation alone in 1 case. Thus of the 6 cases with valid CD4+ T cell data on hCG preparation alone, 1 manifested a significant response. No patient experienced an adverse fall in CD4+ T cell on hCG preparation therapy, although patient PH-VE experienced an 0.7 log rise in viral load with a sustained 50% fall in CD4+ T cell numbers and a partial anti KS response (Figures 2G and H). Similarly, paient PG-17 experienced a significant rise in CD4+ T cells and no change in viral load on hCG therapy alone, yet experienced progression of KS after 2.5 months (Figures 2I and J). All CD4+ T cell values (except for 2 patients on hCG alone) were at or above baseline, with the most significant rises in those on concomitant stable protease inhibitor or non protease drugs (Figure 8B). There is no correlation between a change in the CD4+ T cells count and the dosage of hCG ($r=.101$, $p=.339$, $N=92$) (data not shown).

Among the 26 patients analyzable for weight gain (patients who started hCG preparation therapy coincident with or shortly after starting other anti-viral therapy were excluded), 14 gained weight, 3 experienced weight loss, and 9 remained stable. There was no correlation between weight change and dosage of hCG (data not shown). There was however a pattern observed in some patients where an initial weight gain was followed by a return to baseline levels while others experienced sustained weight gain over several months.

hCG therapy was well tolerated clinically by patients and there was no evidence for an adverse effect of hCG on viral load or CD4+ T cell level. In two cases with advanced HIV disease hCG was discontinued because of coincident cholestasis probably due to other medications in one case and opportunistic infections in the other.

7.3. EFFECTS OF hCG AND β-hCG PEPTIDES ON HEMATOPOIESIS

In addition to the typical decline in CD4· T cells, cytopenias can occur in HIV infected people affecting one or more hematopoietic lineages associated with deficient progenitor cell growth. This deficiency is often made worse by some of the anti-viral therapies currently in use. In contrast, hCG preparations do not inhibit hematopoiesis.

The effect of hCG preparations and peptides was assayed on hematopoietic progenitor cells *in vitro*. Hematopoietic progenitor cells (2×10^5 cell/ml) were isolated from normal bone marrow and cord blood and seeded in methylcellulose. The following hCG preparations and peptides were used in these clonogenic assays were: hCG (APL™); hCG alpha subunit (Sigma); purified hCG heterodimer CR 127; β-hCG peptide 109-119 (SEQ ID NO:7) (Bachem); β-hCG peptide 45-57 (SEQ ID NO:6); β-hCG peptide 45-57 circularized (44-57 with cysteine substituted for the amino acid at position 44, SEQ ID NO:26); mixture of scrambled β-hCG peptides 45-57 and 109-119; and crude preparation of native β-hCG. Also tested were the peptides 45-57 linked at the C-terminus via a peptide bond to the N-terminus of 109-119 (45-57::109-119; SEQ ID NO:30), 47-55 (SEQ ID NO:20) and 48-56 (SEQ ID NO:35) as well as other hCG, α-hCG and β-hCG preparations and α-hCG and β-hCG peptides (Table 2). The hCG preparations were administered at 200 IU/ml and the β- and α- subunits and peptides were administered at 100 μg/ml. The native commercial preparation of hCG (APL™, Wyeth Ayerst) was pre-tested for anti-HIV and anti-KS activities. Aggregates containing more than 50 cells after 10 days of culture were counted as colonies.

As shown in Figures 3A-C and in Table 2, the growth of hematopoietic progenitors (Lunardi-Iskandar et al., 1989, *Leukemia Res.* 13:573-581) is directly promoted by a commercial preparation of partially purified hCG (APL™, Wyeth Ayerst), partially purified native β-chain, and by the synthetic peptides, β-hCG peptide 45-57 (SEQ ID NO:6), β-hCG peptide 109-119 (SEQ ID NO:7), circularized 44-57 with cysteine substituted for the amino acid at position 44 (SEQ ID NO:26), the peptide 45-57 linked at the C-terminus via a

peptide bond to the N-terminus of 109-119 (45-57::109-119; SEQ ID NO:30), and a mixture of the β-hCG peptides 45-57 and 109-119 (SEQ ID NOS:6 and 7, respectively), but not by the highly purified hCG heterodimer (CR127) nor by the recombinant hCG β-chain or the α-chain preparations. The β-hCG peptides 47-55 and 48-56 (SEQ ID NOS:20 and 35, respectively) also exhibited a pro-hematopoietic effect (Table 2). Additionally, scrambled β-hCG peptides 45-57 and 109-119 as well as other β-hCG peptides showed little inhibition (Table 2). Thus, these results recapitulate the anti-KS and anti-HIV effects. Each activity is chiefly effected by the satellin peptides (45-57 and 109-119; SEQ ID NOS:6 and 7, respectively). A series of other peptides of the α- and β-chain had no effect (data not shown).

Table 2

ACTIVITIES OF hCG AND hCG SUBUNIT PREPARATIONS AND hCG PEPTIDES.

Sources	HIV in vitro	HIV transgenic mice	KS in vitro	KS in vivo	Pro-hematopoiesis in vitro
	Inhibition				Enhancement
hCG preparations					
APL™	+++	+++	+++	+++	+++
PREGNYL™	++	++	++	++	++
ORGANON	-	ND	-	-	ND
PROFASI™	+	ND	+	+	+
GOLDLINE	+	ND	-	-	-
STERIS™	++	ND	++	ND	++
SHEIN	+	ND	-	-	-
SIGMA	+++	+++	+++	+++	+++
SIGMA[2]	-	ND	-	-	-
CR127	-	ND	-	ND	-
CR1XY17V	-	ND	-	ND	-
CR1XY17B	-	ND	-	-	-
rhCG	-	-	-	-	-
hCG subunits					
α Chain					
αhCG	-	-	-	-	-

Sources	HIV in vitro	HIV transgenic mice	KS in vitro	KS in vivo	Pro-hematopoiesis in vitro
		Inhibition			Enhancement
rαhCG	-	-	-	-	-
αfp1769A	-	ND	-	ND	-
β Chain					
rβhCG	-	-	-	-	-
βhCG	++	++	++	++	++
Synthetic peptides β-chain hCG					
1. 109-119	+	ND	+	+	+
2. 109-145	+	ND	+	+	+
3. 45-57	++	++	++	++	++
4. Circ 44-57	+++	+++	+++	+++	+++
5. 47-57::108-119	++	++	++	+++	ND
6. 45-57::109-119	++	++	++	++	++
7. 45-57+109-119	++	ND	++	ND	++
8. 41-54	-	-	-	-	-
9. 38-57	-	ND	-	-	-
10. Scrambled 45-57::109-119	-	-	-	-	-
11. Scrambled 45-57	++	ND	++	ND	ND

Sources	HIV	HIV transgenic mice	KS	KS	Pro-hematopoiesis
	in vitro		in vitro	in vivo	in vitro
		Inhibition			Enhancement
12. Scrambled circ. 44-57	-	ND	-	ND	-
13. 6-16	-	-	-	-	-
14. 1-20	-	ND	-	ND	ND
15. 20-47	—	ND	—	ND	—
16. 31-50	-	ND	-	ND	-
17. 46-65	+	ND	+	ND	ND
18. 91-112	ND	ND	-	ND	-
19. 93-100	-	ND	-	ND	ND
20. 110-145	ND	ND	-	ND	-
21. 74-95	-	ND	-	ND	-
22. 7-40	+	ND	+	ND	-
23. 57-93	-	ND	-	ND	-
24. 34-39	-	ND	-	ND	ND
25. 123-145	-	ND	-	ND	ND
26. 134-144	-	ND	-	ND	-
27. 100-110	-	ND	-	ND	ND
28. 113-132	ND	ND	-	ND	-
29. 128-145	-	ND	-	ND	-V

Sources	HIV in vitro	HIV transgenic mice Inhibition	KS in vitro	KS in vivo	Pro-hematopoiesis in vitro Enhancement
30. 37-55	+	+	+	+	+
31. 51-59	-	ND	-	ND	-
32. 48-56	+	+	+	+	+
33. Trimers	-	ND	-	ND	-
Synthetic peptides α-chain hCG					
34. 88-92	-	ND	-	ND	ND
35. 1-15	—	ND	-	-	-
36. 16-30	-	ND	-	ND	-
37. 26-45	-	ND	-	ND	-
38. 41-61	-	ND	-	ND	ND
39. 57-76	-	ND	-	ND	ND
40. 72-92	-	ND	-	ND	-
41. 1-95	-	-	-	-	-

In Table 2, "-" represents less than 10% effect; "+" represents greater than 15% effect; "++" represents greater than 40% effect; "+++" represents greater than 70% effect; and "ND" represents no data available. The "HIV: in vitro" column reports results from assays of the inhibition of HIV-1 replication in vitro (HIV-1 strains and HIV-1 primary isolates). The "HIV transgenic mice" column reports data from the inhibition of HIV RNA and protein expression in HIV-1 transgenic mice. Columns labeled "KS:in vitro" and "KS:in vivo" report on the inhibition of Kaposi's Sarcoma cell growth in vitro in cultured cells and of Kaposi's Sarcoma induced in mice, respectively. Column 5 provides data on the relative increase of hematopoietic colony cell number in vitro clonogenic assays as described above. The commercial hCG preparations tested were APL™ (Wyeth Ayerst), PREGNYL™ (Organon), ORGANON (a highly purified preparation provided by Organon), PROFASI™ (Serono), Goldline, STERIS™, and Shein, and two preparations from Sigma, Sigma[1] (GHO) and Sigma[2] (C1063). The hCG preparations CR127 and CR1XY17V are highly purified hCG preparations and CR1XY17B is a mixture of highly purified α-hCG and β-hCG, all three preparations were obtained from the National Institute of Child Health and Human Development (NICHD) at the National Institute of Health (NIH) and the rhCG is recombinant hCG expressed in a mouse cell line (Sigma). For the hCG subunits "αhCG" and "βhCG" are purified native subunits (Sigma); "rαhCG" and "rβhCG" all the recombinant subunits expressed in mouse cells (Sigma); and αfp1769A is purified, native α subunit (NICHD, NIH). The peptide "scrambled A1" has the sequence Cys-Val-Ala-Gln-Pro-Gly-Pro-Gln-Val-Leu-Leu-Val-Leu-Cys (SEQ ID NO:36) and "Scrambled A2" has the sequence Cys-Val-Ala-Gln-Gly-Val-Leu-Pro-Ala-Leu-Pro-Gln-Val-Val-Cys (SEQ ID NO:37). "Scrambled A1/B" has the sequence of the β-hCG peptides 45-57 (SEQ ID NO:6) and 109-119 (SEQ ID NO;7) which has been scrambled. "Trimers" is a mixture of tripeptides from the β-hCG sequence of amino acids 45-57: Leu-Gln-Gly, Leu-Gln-Pro, Gln-Gly-Val, Gln-Pro-Val, Gln-Val-Leu, Val-Leu-Pro, Leu-Pro-Ala, Leu-Pro-

Pro, Pro-Ala-Leu, Pro-Pro-Leu, Ala-Leu-Pro, Pro-Leu-Pro, Leu-Pro-Gln, Pro-Gln-Val, Gln-Val-Val, and Val-Val-Cys (SEQ ID NOS: 38-53, respectively). Peptides were synthesized by Dr. N. Ambulos (University of Maryland Biomedicine Center), Becham (CA) or Peptide Technologies Corp. (Gaithersburg, MD).

7.4. DISCUSSION

Considerable pro-hematopoietic activity was found with the native partially purified hCG heterodimer and whole β-chain, however, variability in the pro-hematopoietic effect was observed for different hCG preparations and no pro-hematopoietic activity was observed with highly purified (to homogeneity) hCG heterodimer *in vitro*. The lower molecular weight species may retain the pro-hematopoietic effect and that some purification procedure may not eliminate those species.

The available clinically used native hCG and native β-chain preparations are not homogenous and may be contaminated with one or more other active molecules. In this respect, it is noteworthy that though the effects of some preparations of hCG described here were obtained with two different commercial sources of hCG (APL and Pregnyl), one was usually more active (APL) at lower concentrations than any other preparation. The differences in activities of commercial preparations might be explained by variation in the amount of β-hCG fragments. This could be the consequence of different methods of preparation or different sources of human urine. For example, free β is more abundant in the earliest weeks of pregnancy. Consequently, we initiated studies with a variety of synthetic peptides, and our results show that all the *in vitro* activities of the preparations of native hCG are mimicked by the β-hCG peptides 45-57 and 109-119 but not other β- or α-peptides or scrambled 45-57 peptide. Thus, β-hCG may contain structural motifs that produce effects which probably work by mechanisms which differ from those currently known for hCG, and β-hCG peptides may have biological

functions quite distinct from the conventional effects of the heterodimer.

8. EXAMPLE: FRACTIONATION OF ACTIVE hCG PREPARATIONS AND HUMAN EARLY PREGNANCY URINE

The present inventors have found that certain commercial preparations of hCG, for example, hCG APL™ (Wyeth-Ayerst), had higher anti-HIV, anti-Kaposi's Sarcoma, anti-wasting and pro-hematopoietic activity than other commercial preparations of hCG (see discussion in section 7 *supra*). Further, the inventors have also shown that highly purified preparations of native and recombinant hCG and β-hCG had no activity against HIV infection or replication or against Kaposi's Sarcoma (see results discussion section 7 *supra*). Accordingly, the inventors postulated that there must be an activity in the hCG commercial preparations that is not the hCG dimer or the β-hCG subunit, responsible for the anti-HIV, anti-Kaposi's Sarcoma, anti-wasting and pro-hematopoietic activities. This section presents results of the fractionation of the APL™ hCG commercial preparation and urine from women in the first trimester of pregnancy ("human early pregnancy urine") which also contains hCG. Particular sizing column chromatography fractions were shown to have activity, thus demonstrating that the active components could be fractionated.

8.1. MATERIALS AND METHODS

Both human early pregnancy urine and the APL™ (Wyeth-Ayerst) hCG commercial preparation were subjected to fractionation. For the human early pregnancy urine, 5 liters of urine were collected from women in the first trimester of pregnancy. Twenty-four hour collections were stored frozen or refrigerated for up to 2 days. Upon delivery of the urine to the laboratory, sodium azide was added at 1 g/liter and the urine frozen until five liters had been collected. At this time, all the urine was thawed overnight, and the pH was

adjusted to 7.2-7.4 with NaOH, which causes some precipitation. The precipitate was allowed to sediment for 1 hour at room temperature, most of the supernatant decanted and the remaining supernatant centrifuged to remove any additional precipitate with that supernatant being added to the first supernatant decanted. Next, the urine was concentrated with a Pellicon (Millipore) filtration system using a membrane cassette with a 3,000 MW cut off, which concentrates the urine approximately 60 to 80 fold. Next, the urine was desalted and delipidated by passing 500 ml of the material at a time through a Sephadex G25 column with a volume of 1.7 liters in 0.05 M ammonium bicarbonate (the column was washed between runs with 25% ethanol to remove absorbed lipids and glycoprotein). The material was lyophilized and stored for further fractionation. The urinary material was then reconstituted in 6 ml of 30 mM sodium phosphate buffer, pH 8.3 fractionation.

For the APL™ hCG, the lyophilized hCG preparation from eleven vials (each vial containing 20,000 IU hCG) was resuspended in 6 ml of 30 mM sodium phosphate buffer, pH 8.3 and filtered twice through the 0.45 μm particle filter. For both the lyophilized urine and the hCG APL™, the prepared sample was then loaded onto a pre-packed SUPERDEX™ 200 HiLoad Column (Pharmacia 26 mm^2 x 60 cm) in the 30 mM sodium phosphate buffer, pH 8.3 and then eluted from the column with a solution containing 30 mM sodium phosphate buffer, pH 7.0 and 2 M NaCl. For the first ten minutes, the column flow rate was 1 ml/minute (due to the viscosity of the hCG APL™ material; this flow rate scheme was also used for the urine material); after the first 10 minutes, the flow rate was 2 ml/minute. The column was run on a Hewlett Packard 1050 HPLC equipped with a photodiode array detector. Four ml fractions were collected and frozen until further analysis.

The protein concentration in each fraction was determined by amino acid analysis. A 50 μl aliquot of alternate column fractions was processed for anlysis by hydrolysis in vapors of 6N HCl with 0.1% phenol at 110°C for

24 hours in a Waters Associates Pico-Tag Workstation (Waters, Milford, MA). An internal standard, norleucine, was added to all fraction samples before hydrolysis to correct for any losses during hydrolysis or liquid transfer. The hydrolyzed samples were then analyzed on a Beckman Instruments 6300 amino acid analyzer and the data was collected on the PE Nelson Data System (Perkin-Elmer) and transformed using PE Nelson Turbochrome software.

The column fractions were monitored with immunoassays to heterodimeric hCG as well as to the hCG beta core fragment (O'Connor et al., 1994, *Endocrin. Rev* 15:650-683; Krichevsky et al., 1994, *Endocrinology* 134:1139-145; Krichevsky et al., 1991, *Endocrinology* 128:1255-1264; O'Connor et al., 1988, *Cancer Res.* 48:1361-1366; Krichevsky, 1988, *Endocrinology* 128:584-593). These two assays permit placement of two internal standard sizes for the gel filtration column: 70,000 kD (hCG) and 10,000 kD (hCG beta core fragment which is amino acids 6-40 of β-hCG linked via a disulfide bond to amino acids 55-92 of β-hCG). External molecular weight standards were also employed to calibrate the column elution positions. In addition, MALDI-TOF mass spectrometry was used to evaluate the ions observed in certain active fractions. Mass spectrometry did indicate that some peptides separated at anomalous positions, showing that they were being carried by other proteins to earlier elution positions in some cases, or interacting with the column matrix and eluting much later than their molecular size would indicate. For example, 3,000 and 6,000 molecular weight materials eluted from the gel filtration column with material of 14,000 molecular weight while 11,000 molecular weight material eluted with material of approximately 1,000-2,000 molecular weight, hCG and hCG-related molecules eluted at their expected positions.

The fractions were then tested for anti-HIV, anti-KS and pro-hematopoietic activities in vitro. To assay for inhibition of HIV-1 replication in vitro, the HIV-1 IIID viral strain was used to infect PBMCs and PM-1 cells (derived from the HUT-78 T-cell lymphoma cell line) at 10^3 $TCID_{50}$/ml.

The infected cells were incubated for three days in 100 IU/ml of the hCG APL™ or β-hCG C-Sigma preparations; 50-100 μl per ml of the hCG APL™ or early pregnancy urine fractions; 50 μg/ml β-core protein or α-hCG preparation; 200 IU/ml of the highly purified CR127 hCG preparation; or 100 μl/ml of the circularized β-hCG peptide 44-57 (with cysteine substituted at position 44; SEQ ID NO:26). The level of HIV was determined by measurement of p24 antigen levels.

To assay for activity against Kaposi's sarcoma cell growth in vitro, an *in vitro* clonogenic assay with cultured Kaposi's Sarcoma cells was used (Lunardi-Iskandar et al., 1995, *Nature* 375:64-68; Nakamura et al., 1988, *Science* 242:426-430; Ensoli et al., 1989, *Science* 243:223-226; Salahuddin et al., 1988, *Science* 242:430-433; Masood, et al., 1984, *AIDS Res. Hum. Retroviruses* 10:969-976). Briefly, the KS Y-1 cells were obtained from mononuclear cells isolated from pleural effusion of an AIDS patient with KS in the lungs. After the depletion of T lymphocytes, monocytes/macrophages and fibroblasts using monoclonal antibodies against CD2, CD3, CD4, CD8, CD10 and CD14 membrane antigens and baby rabbit complement, the cells were cultured in the absence of exogenous growth factors to select for transformed cells. Immunological characterization of the KS Y-1 cells showed that CD34, CD31 and endoglin were expressed. Clonogenic assays were performed by seeding the KS Y-1 or KS-SLK cells in methylcellulose (0.8%, v/v), incubating the cells for 10 days in the presence or absence of the test substance and then counting the number of well-formed colonies of triplicate wells formed after seeding with 5 X 10^4 cells.7.7 *supra* was used with the KS Y-1 and KS-SLK cultured Kaposi's Sarcoma cell lines. The cells were incubated in 200 IU/ml of commercial hCG preparations; 50 μl/ml of certain fractions from the hCG preparation of early pregnancy urine fractionation; or 100 μg/ml β- and α-hCG chains, β-hCG core protein, β-hCG peptides or LH (leuteinizing hormone).

Pro-hematopoietic activity was assayed in in-vitro clonogenic assays as described in Section 7.3 *supra*. Cells

were assayed for colony formation in the presence of 200 IU/ml hCG APL™ or highly purified hCG preparation CR 127; 100 μl/ml of the fractions of the hCG commercial preparation of early pregnancy urine; or 100 μg/ml β-hCG core protein or cyclized β-hCG peptide of amino acids 44-57 (with cysteine substituted at position 44; SEQ ID NO:26).

The unfractionated APL™ hCG preparation, PREGNYL™ (Organon) hCG preparation, purified β-core and phenol were also tested in certain assays. Phenol, which is an additive in the hCG APL™ preparation, was tested to control for any effect on cell growth or viral inhibition.

8.2. RESULTS

Fractionation of both the APL™ hCG preparation and the human early pregnancy urine resulted in a significant protein peak at approximately 158 kD with diminishing, but still detectable, protein in the rest of the fractions, even those containing small molecular weight material (Figures 6A and D). Fractions containing the hCG dimer (77 kD) and the β-hCG core (10 kD) were identified by immunoprecipitation using antibodies that specifically recognize these particular species, as described in the materials and methods section 8.1. The elution profile of the commercial hCG material was also plotted in comparison to the elution of standard molecular weight markers (Figures 9A and B). Additionally, Fractions 61, 63, 64, 65 and 67 from the fractionation of the commercial hCG material was analyzed by MALDI-TOF mass spectrometry (Figures 10A-E, respectively).

8.2.1. EFFECT OF FRACTIONS OF COMMERCIAL hCG PREPARATIONS AND EARLY PREGNANCY URINE ON HIV-1 REPLICATION IN VITRO

The fractions of both the APL™ hCG preparation and the human early pregnancy urine were assayed for inhibition of HIV-1 IIID replication in PBMCs and PM-1 cells as described above. Many of the APL™ hCG preparation fractions exhibited significant inhibition of HIV-1 IIID replication (Figure 6C).

In particular, fractions containing material of approximately 70 kD to approximately 2-3 kD exhibited HIV-1 inhibitory activity. The fractions effecting the highest percent inhibition of HIV-1 replication were fractions 62, 63, 65, and 73, with the three main peaks of activity eluting with apparent molecular weights of approximately 40 kD, approximately 15 kD, and approximately 2-3 kD, as determined by comparison with the elution of hCG (77 kD) and β-core protein (10 kD).

The fractions of human early pregnancy urine were also assayed for ability to inhibit HIV-1 IIID replication in the PBMCs and the PM-1 cells. Again, several fractions had at least some HIV-1 replication-inhibitory activity. Fractions 64 and 67 caused more than twice the inhibition of HIV-1 IIID replication than any of the other fractions (Figure 6F). There were approximately two peaks of activity eluting from the gel filtration column with apparent molecular weights of approximately 15 kD and 3 kD, as determined by comparison with the elution of hCG (77 D) and β-core protein (10 kD) identified by immunoassay.

Additionally, phenol had no effect on HIV-1 replication, demonstrating that the anti-HIV activity of the APL™ hCG is not due to the presence of phenol in the APL™ hCG preparation, and purified β-hCG core protein (the peptide of amino acids to 6-40 of β-hCG linked via a disulfide bond to the peptide of amino acids 55-92 of β-hCG as depicted in Figure 8 (SEQ ID NO:2)) was also found not to inhibit HIV-1 replication (data not shown).

8.2.2. EFFECT OF FRACTIONS OF COMMERCIAL hCG AND EARLY PREGNANCY URINE ON KAPOSI'S SARCOMA CELL GROWTH IN VITRO

The fractions of APL™ hCG and human early pregnancy urine were also tested for inhibition of the proliferation of cultured Kaposi's Sarcoma cells. Figure 6B depicts the results of assays of the APL™ hCG fractions for inhibition of KS Y-1 cell growth. There were three major peaks of KS cell

growth inhibitory activity which eluted from the gel filtration column with apparent molecular weights of approximately 40 kD, approximately 15 kD, and approximately 2-3 kD, as compared with the elutions of fractions containing hCG dimer (77 kD) and β-core protein (10 kD). A fraction containing material about the same size as the β-hCG core protein exhibited the highest level of inhibition; however, purified β-hCG core was found not to inhibit KS cell growth (data not shown).

Fractions of human early pregnancy urine were also assayed for inhibition of KS Y-1 cell growth. Fractions containing material which eluted from the gel filtration column with apparent molecular weights of approximately 15 kD and approximately 2-3 kD as compared with the elution of fractions containing hCG dimer (77 kD) and the β-hCG core (10 kD) as identified by immunoprecipitation assay were the most effective at inhibiting KS cell growth, with the approximately 15 kD fractions having the highest activity (Figure 6E).

8.2.3. EFFECT OF COMMERCIAL hCG AND EARLY PREGNANCY URINE FRACTIONS ON HEMATOPOIESIS IN VIVO

Figure 7 presents data on the effect of hCG and hCG-related preparations on hematopoiesis in in vitro clonogenic assays for numbers of colony forming units of granulocytes, erythrocytes, megakaryocytes and monocytes (CFU-GEMM), burst forming units of erythrocytes (BFU-e) and colony forming units of granulocytes and macrophages (CFU-GM). Figure 7 shows that fraction 65 of both the hCG APL™ and early pregnancy urine fractionation (bars 7 and 8, respectively; fraction 65 contains material with an apparent molecular weight of approximately 15 kD) promoted hematopoiesis in all three assays. Fraction 26 of the early pregnancy urine fractionation (bar 9) did not promote hematopoiesis in any of the assays. The purified β-core protein (bar 10) likewise exhibited no stimulation of hematopoiesis.

These results also confirm that the hCG APL™ preparation, native β-hCG and the cyclized β-hCG peptide of amino acids 44-57 (cysteine substituted at position 44; SEQ ID NO: 26) (bars 3, 5 and 6, respectively) all have pro-hematopoietic activity. The α-subunit of hCG, highly purified hCG preparation CR127 and PBS alone (bars 2, 4 and 1, respectively) did not promote hematopoiesis.

8.3. CONCLUSION

The above-described experiments demonstrate that the factor(s) responsible for the anti-HIV and anti-KS activities can be further isolated from the hCG preparations by gel filtration on a SUPERDEX™ 200 gel filtration column. The factor(s) were fractionated from both the commercial APL™ hCG preparation and urine from women in early pregnancy (first trimester). The fractions of highest anti-HIV and anti-KS activity contained material eluting from the gel filtration column with an apparent molecular weights of approximately 40 kD, 15 kD and 2-3 kD. Although certain active fractions contained material of approximately the size of the β-hCG core protein (~10 kD), purified β-hCG core protein was found to have neither anti-HIV nor anti-KS activity. The fractions exhibiting anti-HIV and anti-KS activity in vitro also had pro-hematopoietic activity in vitro. Furthermore, phenol, an additive in the APL™ hCG preparation, had no anti-HIV activity.

The present invention is not to be limited in scope by the specific embodiments described herein. Indeed, various modifications of the invention in addition to those described herein will become apparent to those skilled in the art from the foregoing description and accompanying figures. Such modifications are intended to fall within the scope of the appended claims.

Various references are cited herein, the disclosures of which are incorporated by reference in their entireties.

SEQUENCE LISTING

(1) GENERAL INFORMATION

(i) APPLICANT: Gallo, Robert C.
Bryant, Joseph
Lunardi-Iskandar, Yanto

(ii) TITLE OF THE INVENTION: METHODS OF PROMOTING HEMATOPOIESIS USING DERIVATIVES OF HUMAN CHORIONIC GONADOTROPIN

(iii) NUMBER OF SEQUENCES: 37

(iv) CORRESPONDENCE ADDRESS:
(A) ADDRESSEE: Pennie & Edmonds
(B) STREET: 1155 Avenue of the Americas
(C) CITY: New York
(D) STATE: NY
(E) COUNTRY: USA
(F) ZIP: 10036/2711

(v) COMPUTER READABLE FORM:
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(viii) ATTORNEY/AGENT INFORMATION:
(A) NAME: Misrock, S. Leslie
(B) REGISTRATION NUMBER: 18,872
(C) REFERENCE/DOCKET NUMBER: 8769-026

(ix) TELECOMMUNICATION INFORMATION:
(A) TELEPHONE: 212-790-9090
(B) TELEFAX: 212-869-8864
(C) TELEX: 66141 PENNIE

(2) INFORMATION FOR SEQ ID NO:1:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 539 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA

(ix) FEATURE:
(A) NAME/KEY: CDS
(B) LOCATION: 26..520

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

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AGACAAGGCA GGGGACGCAC CAAGG ATG GAG ATG TTC CAG GGG CTG CTG CTG          52
                            Met Glu Met Phe Gln Gly Leu Leu Leu
                            -20                 -15

TTG CTG CTG CTG AGC ATG GGC GGG ACA TGG GCA TCC AAG GAG CCG CTT          100
Leu Leu Leu Leu Ser Met Gly Gly Thr Trp Ala Ser Lys Glu Pro Leu
    -10                 -5                  1

CGG CCA CGG TGC CGC CCC ATC AAT GCC ACC CTG GCT GTG GAG AAG GAG          148
Arg Pro Arg Cys Arg Pro Ile Asn Ala Thr Leu Ala Val Glu Lys Glu
                10                  15                  20

GGC TGC CCC GTG TGC ATC ACC GTC AAC ACC ACC ATC TGT GCC GGC TAC          196
Gly Cys Pro Val Cys Ile Thr Val Asn Thr Thr Ile Cys Ala Gly Tyr
            25                  30                  35

TGC CCC ACC ATG ACC CGC GTG CTG CAG GGG GTC CTG CCG GCC CTG CCT          244
Cys Pro Thr Met Thr Arg Val Leu Gln Gly Val Leu Pro Ala Leu Pro
        40                  45                  50

CAG GTG GTG TGC AAC TAC CGC GAT GTG CGC TTC GAG TCC ATC CGG CTC          292
Gln Val Val Cys Asn Tyr Arg Asp Val Arg Phe Glu Ser Ile Arg Leu
    55                  60                  65

CCT GGC TGC CCG CGC GGC CTG AAC CCC GTG GTC TCC TAC GCC GTG GCT          340
Pro Gly Cys Pro Arg Gly Leu Asn Pro Val Val Ser Tyr Ala Val Ala
70                  75                  80                  85

CTC AGC TGT CAA TGT GCA CTC TGC CGC CGC AGC ACC ACT GAC TGC GGG          388
Leu Ser Cys Gln Cys Ala Leu Cys Arg Arg Ser Thr Thr Asp Cys Gly
                90                  95                  100

GGT CCC AAG GAC CAC CCC TTG ACC TGT GAT GAC CCC CGC TTC CAG GAC          436
Gly Pro Lys Asp His Pro Leu Thr Cys Asp Asp Pro Arg Phe Gln Asp
            105                 110                 115

TCC TCT TCC TCA AAG GCC CCT CCC CCC AGC CTT CCA AGC CCA TCC CGA          484
Ser Ser Ser Ser Lys Ala Pro Pro Pro Ser Leu Pro Ser Pro Ser Arg
        120                 125                 130

CTC CCG GGG CCC TCG GAC ACC CCG ATC CTC CCA CAA TAAAGGCTTC               530
Leu Pro Gly Pro Ser Asp Thr Pro Ile Leu Pro Gln
    135                 140                 145

TCAATCCGC                                                               539
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(2) INFORMATION FOR SEQ ID NO:2:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 165 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

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Met Glu Met Phe Gln Gly Leu Leu Leu Leu Leu Leu Ser Met Gly
-20                 -15                 -10                 -5

Gly Thr Trp Ala Ser Lys Glu Pro Leu Arg Pro Arg Cys Arg Pro Ile
                 1                   5                  10

Asn Ala Thr Leu Ala Val Glu Lys Glu Gly Cys Pro Val Cys Ile Thr
         15                  20                  25

Val Asn Thr Thr Ile Cys Ala Gly Tyr Cys Pro Thr Met Thr Arg Val
      30                  35                  40

Leu Gln Gly Val Leu Pro Ala Leu Pro Gln Val Val Cys Asn Tyr Arg
 45                  50                  55                  60

Asp Val Arg Phe Glu Ser Ile Arg Leu Pro Gly Cys Pro Arg Gly Leu
                 65                  70                  75

Asn Pro Val Val Ser Tyr Ala Val Ala Leu Ser Cys Gln Cys Ala Leu
             80                  85                  90

Cys Arg Arg Ser Thr Thr Asp Cys Gly Gly Pro Lys Asp His Pro Leu
         95                 100                 105

Thr Cys Asp Asp Pro Arg Phe Gln Asp Ser Ser Ser Ser Lys Ala Pro
     110                 115                 120

Pro Pro Ser Leu Pro Ser Pro Ser Arg Leu Pro Gly Pro Ser Asp Thr
125                 130                 135                 140

Pro Ile Leu Pro Gln
                145
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(2) INFORMATION FOR SEQ ID NO:3:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 14 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

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Met Thr Arg Val Leu Gln Gly Val Leu Pro Ala Leu Pro Gln
1               5                  10
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(2) INFORMATION FOR SEQ ID NO:4:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 10 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:

Leu Gln Gly Val Leu Pro Ala Leu Pro Gln
1 5 10

(2) INFORMATION FOR SEQ ID NO:5:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 7 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:5:

Gly Val Leu Pro Ala Leu Pro
1 5

(2) INFORMATION FOR SEQ ID NO:6:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 13 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:6:

Leu Gln Gly Val Leu Pro Ala Leu Pro Gln Val Val Cys
1 5 10

(2) INFORMATION FOR SEQ ID NO:7:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 11 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: circular, linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:7:

Thr Cys Asp Asp Pro Arg Phe Gln Asp Ser Ser
1 5 10

(2) INFORMATION FOR SEQ ID NO:8:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 13 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:8:

Met Thr Arg Val Leu Gln Gly Val Leu Pro Ala Leu Pro
1 5 10

(2) INFORMATION FOR SEQ ID NO:9:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 12 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:9:

Thr Arg Val Leu Gln Gly Val Leu Pro Ala Leu Pro
1 5 10

(2) INFORMATION FOR SEQ ID NO:10:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 11 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:10:

Arg Val Leu Gln Gly Val Leu Pro Ala Leu Pro
1 5 10

(2) INFORMATION FOR SEQ ID NO:11:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 10 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:11:

Val Leu Gln Gly Val Leu Pro Ala Leu Pro
1 5 10

(2) INFORMATION FOR SEQ ID NO:12:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 14 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:12:

Val Leu Gln Gly Val Leu Pro Ala Leu Pro Gln Val Val Cys
1 5 10

(2) INFORMATION FOR SEQ ID NO:13:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 9 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:13:

Leu Gln Gly Val Leu Pro Ala Leu Pro
1 5

(2) INFORMATION FOR SEQ ID NO:14:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 8 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:14:

Gln Gly Val Leu Pro Ala Leu Pro
1 5

(2) INFORMATION FOR SEQ ID NO:15:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 10 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:15:

Leu Gln Gly Val Leu Pro Ala Leu Pro Gln
1 5 10

(2) INFORMATION FOR SEQ ID NO:16:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 11 amino acids

(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:16:

Leu Gln Gly Val Leu Pro Ala Leu Pro Gln Val
1 5 10

(2) INFORMATION FOR SEQ ID NO:17:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 12 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:17:

Leu Gln Gly Val Leu Pro Ala Leu Pro Gln Val Val
1 5 10

(2) INFORMATION FOR SEQ ID NO:18:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 14 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:18:

Leu Gln Gly Val Leu Pro Ala Leu Pro Gln Val Val Cys Asn
1 5 10

(2) INFORMATION FOR SEQ ID NO:19:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 8 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:19:

Gly Val Leu Pro Ala Leu Pro Gln
1 5

(2) INFORMATION FOR SEQ ID NO:20:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 9 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:20:

Gly Val Leu Pro Ala Leu Pro Gln Val
1 5

(2) INFORMATION FOR SEQ ID NO:21:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 10 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:21:

Gly Val Leu Pro Ala Leu Pro Gln Val Val
1 5 10

(2) INFORMATION FOR SEQ ID NO:22:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 12 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:22:

Gly Val Leu Pro Ala Leu Pro Gln Val Val Cys Asn
1 5 10

(2) INFORMATION FOR SEQ ID NO:23:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 98 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:23:

Val Leu Pro Ala Leu Pro Gln Val Val Cys Asn Tyr Arg Asp Val Arg
1 5 10 15

Phe Glu Ser Ile Arg Leu Pro Gly Cys Pro Arg Gly Leu Asn Pro Val
20 25 30

Val Ser Tyr Ala Val Ala Leu Ser Cys Gln Cys Ala Leu Cys Arg Arg
35 40 45

Ser Thr Thr Asp Cys Gly Gly Pro Lys Asp His Pro Leu Thr Cys Asp
50 55 60

Asp Pro Arg Phe Gln Asp Ser Ser Ser Ser Lys Ala Pro Pro Pro Ser
65 70 75 80

Leu Pro Ser Pro Ser Arg Leu Pro Gly Pro Ser Asp Thr Pro Ile Leu
85 90 95

Pro Gln

(2) INFORMATION FOR SEQ ID NO:24:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 88 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:24:

Asn Tyr Arg Asp Val Arg Phe Glu Ser Ile Arg Leu Pro Gly Cys Pro
1 5 10 15

Arg Gly Leu Asn Pro Val Val Ser Tyr Ala Val Ala Leu Ser Cys Gln
20 25 30

Cys Ala Leu Cys Arg Arg Ser Thr Thr Asp Cys Gly Gly Pro Lys Asp
35 40 45

His Pro Leu Thr Cys Asp Asp Pro Arg Phe Gln Asp Ser Ser Ser Ser
50 55 60

Lys Ala Pro Pro Pro Ser Leu Pro Ser Pro Ser Arg Leu Pro Gly Pro
65 70 75 80

Ser Asp Thr Pro Ile Leu Pro Gln
85

(2) INFORMATION FOR SEQ ID NO:25:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 37 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:25:

Thr Cys Asp Asp Pro Arg Phe Gln Asp Ser Ser Ser Ser Lys Ala Pro
1 5 10 15

Pro Pro Ser Leu Pro Ser Pro Ser Arg Leu Pro Gly Pro Ser Asp Thr
20 25 30

Pro Ile Leu Pro Gln
35

(2) INFORMATION FOR SEQ ID NO:26:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 14 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: circular

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:26:

Cys Leu Gln Gly Val Leu Pro Ala Leu Pro Gln Val Val Cys
1 5 10

(2) INFORMATION FOR SEQ ID NO:27:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 10 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:27:

Cys Asp Asp Pro Arg Phe Gln Asp Ser Ser
1 5 10

(2) INFORMATION FOR SEQ ID NO:28:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 11 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:28:

Gly Val Leu Pro Ala Leu Pro Gln Val Val Cys
1 5 10

(2) INFORMATION FOR SEQ ID NO:29:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 12 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:29:

Leu Thr Cys Asp Asp Pro Arg Phe Gln Asp Ser Ser
1 5 10

(2) INFORMATION FOR SEQ ID NO:30:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 24 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:30:

Leu Gln Gly Val Leu Pro Ala Leu Pro Gln Val Val Cys Thr Cys Asp
1 5 10 15
Asp Pro Arg Phe Gln Asp Ser Ser
20

(2) INFORMATION FOR SEQ ID NO:31:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 23 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:31:

Cys Asp Asp Pro Arg Phe Gln Asp Ser Ser Leu Gln Gly Val Leu Pro
1 5 10 15
Ala Leu Pro Gln Val Val Cys
20

(2) INFORMATION FOR SEQ ID NO:32:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 23 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:32:

Gly Val Leu Pro Ala Leu Pro Gln Val Val Cys Leu Thr Cys Asp Asp
1 5 10 15
Pro Arg Phe Gln Asp Ser Ser
20

(2) INFORMATION FOR SEQ ID NO:33:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 34 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:33:

```
Pro Arg Cys Arg Pro Ile Asn Ala Thr Leu Ala Val Glu Lys Glu Gly
 1               5                  10                  15
Cys Pro Val Cys Ile Thr Val Asn Thr Thr Ile Cys Ala Gly Tyr Cys
            20                  25                  30
Pro Thr
```

(2) INFORMATION FOR SEQ ID NO:34:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 20 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:34:

```
Gln Gly Val Leu Pro Ala Leu Pro Gln Val Val Cys Asn Tyr Arg Asp
 1               5                  10                  15
Val Arg Phe Glu
            20
```

(2) INFORMATION FOR SEQ ID NO:35:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 9 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:35:

```
Val Leu Pro Ala Leu Pro Gln Val Val
 1               5
```

(2) INFORMATION FOR SEQ ID NO:36:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 14 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:36:

```
Cys Val Ala Gln Pro Gly Pro Gln Val Leu Leu Val Leu Cys
 1               5                  10
```

(2) INFORMATION FOR SEQ ID NO:37:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 15 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:37:

Cys Val Ala Gln Gly Val Leu Pro Ala Leu Pro Gln Val Val Cys
1 5 10 15

WHAT IS CLAIMED IS:

1. A method of treating or preventing a disease or disorder by increasing the production of one or more hematopoietic cell types in a subject in need of such treatment or prevention comprising administering to the subject an amount of a preparation comprising hCG or β-hCG, effective to increase the production of one or more hematopoietic cell types.

2. The method of claim 1 in which the subject is a human.

3. The method of claim 1 in which the subject has HIV infection, idiopathic thrombocytopenic purpura, anemia or neutropenia.

4. The method of claim 1 in which the subject has undergone chemotherapy or radiation therapy.

5. The method of claim 1 in which at least 15,000 units, or at least 30,000 units, or at least 45,000 units of the preparation is administered per week.

6. A method of treating or preventing a disease or disorder by increasing the production of one or more hematopoietic cell types in a subject in need of such treatment or prevention comprising administering to the subject an amount of a purified protein effective to increase the production of one or more hematopoietic cell types, which protein comprises one or more portions of the amino acid sequence of β-hCG, a peptide having an amino acid sequence consisting of said one or more portions being active to increase the production of one or more hematopoietic cell types.

7. The method of claim 6 in which the amino acid sequence of at least one of said one or more portions is selected from the group consisting of amino acid numbers 41-54, 45-54, 47-53, 45-57, 109-119, 41-53, 42-53, 43-53, 44-53, 44-57, 45-53, 46-53, 45-54, 45-55, 45-56, 45-58, 47-54, 47-57, 47-56, 47-58, 48-145, 58-145, 109-145, 7-40, 46-65, and 48-56 (SEQ ID NOS:3-25 and 33-35, respectively), as depicted in Figure 4 (a portion of SEQ ID NO:2).

8. The method of claim 6 in which the purified protein comprises two or more at least five amino acid, non-naturally contiguous portions of the amino acid sequence of β-hCG as depicted in Figure 4 (SEQ ID NO:2), in which said portions are linked via a peptide bond between the N-terminus of a first said portion and the C-terminus of a second said portion.

9. The method of claim 8, in which the amino acid sequence of said protein is selected from the group consisting of β-hCG amino acids 45-57 (SEQ ID NO:6) linked at the C-terminus via a peptide bond to the N-terminus of β-hCG amino acids 109-119 (SEQ ID NO:7), β-hCG amino acids 110-119 (SEQ ID NO:27) linked at the C-terminus via a peptide bond to the N-terminus of β-hCG amino acids 45-57 (SEQ ID NO:6), and β-hCG amino acids 47-57 (SEQ ID NO:28) linked at the C-terminus via a peptide bond to the N-terminus of β-hCG amino acids 108-119 (SEQ ID NO:29) as depicted in Figure 4 (SEQ ID NO:2).

10. A method of treating or preventing a disease or disorder by increasing the production of one or more hematopoietic cell types in a subject in need of such treatment or prevention comprising administering to the subject an amount of a purified derivative of a protein effective to increase the production of one or more hematopoietic cell types, which protein comprises one or more portions of the amino acid sequence of β-hCG, a peptide

having an amino acid sequence consisting of said one or more portions being active to increase the production of one or more hematopoietic cell types.

11. The method of claim 10 in which the amino acid sequence of at least one of said one or more portions is selected from the group consisting of amino acid numbers 41-54, 45-54, 47-53, 45-57, 109-119, 41-53, 42-53, 43-53, 44-53, 44-57, 45-53, 46-53, 45-54, 45-55, 45-56, 45-58, 47-54, 47-57, 47-56, 47-58, 48-145, 58-145, 109-145, 7-40, 46-65, and 48-56 (SEQ ID NOS:3-25 and 33-35, respectively) as depicted in Figure 4 (a portion of SEQ ID NO:2).

12. The method of claim 10 in which the purified derivative comprises two or more at least five amino acid, non-naturally contiguous portions of the amino acid sequence of β-hCG as depicted in Figure 4 (SEQ ID NO:2), in which said portions are linked via a peptide bond between the N-terminus of a first said portion and the C-terminus of a second said portion.

13. The method of claim 12, in which the amino acid sequence of said derivative is selected from the group consisting of β-hCG amino acids 45-57 (SEQ ID NO:6) linked at the C-terminus via a peptide bond to the N-terminus of β-hCG amino acids 109-119 (SEQ ID NO:7), β-hCG amino acids 110-119 (SEQ ID NO:27) linked at the C-terminus via a peptide bond to the N-terminus of β-hCG amino acids 45-57 (SEQ ID NO:6), and β-hCG amino acids 47-57 (SEQ ID NO:28) linked at the C-terminus via a peptide bond to the N-terminus of β-hCG amino acids 108-119 (SEQ ID NO:29) as depicted in Figure 4 (SEQ ID NO:2).

14. The method of claim 6 or 10 in which the subject is a human.

15. The method of claim 6 or 10 in which the amino acid sequence of the protein consists of amino acid numbers 45-57 (SEQ ID NO:6) as depicted in Figure 4 (a portion of SEQ ID NO:2).

16. The method of claim 6 in which the protein is N-acetylated and has a C-terminal amide.

17. The method of claim 10 in which the derivative contains one or more D-amino acids or one or more non-classical amino acids.

18. The method of claim 6 or 10 in which the subject has HIV infection, idiopathic thrombocytopenic purpura, an anemia or a neutropenia.

19. The method of claim 6 or 10 in which the subject has undergone chemotherapy or radiation therapy.

20. A method of treating or preventing a disease or disorder by increasing the production of one or more hematopoietic cell types in a subject in need of such treatment or prevention comprising administering to the subject an amount of a circularized protein effective to increase the production of one or more hematopoietic cell types, the amino acid sequence of which protein consists of one or more portions of the sequence of β-hCG as depicted in Figure 4 (SEQ ID NO:2) in which a cysteine residue is inserted or substituted for a different amino acid residue in at least one of said one or more portions of said sequence, said one or more portions of said sequence containing a second cysteine residue, and in which a disulfide bond is formed between the inserted or substituted cysteine residue and the second cysteine residue present in said one or more portions of said sequence, said protein being active to increase the production of one or more hematopoietic cell types.

21. The method of claim 20 in which at least one of said one or more portions has an amino acid sequence selected from the group consisting of amino acid numbers 41-54, 45-54, 47-53, 45-57, 109-119, 41-53, 42-53, 43-53, 44-53, 44-57, 45-53, 46-53, 45-54, 45-55, 45-56, 45-58, 47-54, 47-57, 47-56, 47-58, 48-145, 58-145, 109-145, 7-40, 46-65, and 48-56 (SEQ ID NOS:3-25 and 33-35, respectively) as depicted in Figure 4 (a portion of SEQ ID NO:2).

22. The method of claim 20 in which the circularized protein has an amino acid sequence which consists of β-hCG amino acid numbers 44-57 (SEQ ID NO:12) as depicted in Figure 4 (a portion of SEQ ID NO:2), with cysteine substituted for valine at position 44.

23. The method of claim 20 in which said circularized protein consists of two or more at least five amino acid, non-naturally contiguous portions of the sequence of β-hCG as depicted in Figure 4 (SEQ ID NO:2), in which the portions are linked via a peptide bond between the N-terminus of a first said portion and the C-terminus of a second said portion.

24. The method of claim 23, in which the first portion consists of amino acid numbers 45-57 (SEQ ID NO:6) and the second portion consists of amino acid numbers 110-119 (SEQ ID NO:27) as depicted in Figure 4 (SEQ ID NO:2); and in which a disulfide bond is formed between the cysteine residues at a amino acids 57 and 110 of said portions.

25. A method of treating or preventing a disease or disorder by increasing the production of one or more hematopoietic cell types in a subject in need of such treatment or prevention comprising administering to the subject an amount of a purified protein effective to increase the production of one or more hematopoietic cell types, the amino acid sequence of which consists of one or more portions of the sequence of β-hCG as depicted in Figure 4 (SEQ ID

NO:2) in which one or more residues in at least one of said one or more portions of said sequence are substituted by an amino acid or amino acid analog having a side chain with an amino or carboxyl group, said amino or carboxyl group forming a peptide bond with a second sequence of one or more amino acids, said protein being active to increase the production of one or more hematopoietic cell types.

26. The method of claim 25 in which said at least one portion of said sequence consists of amino acid numbers 41-54, 45-54, 47-53, 45-57, 109-119, 41-53, 42-53, 43-53, 44-53, 44-57, 45-53, 46-53, 45-54, 45-55, 45-56, 45-58, 47-54, 47-57, 47-56, 47-58, 48-145, 58-145, 109-145, 7-40, 46-65, and 48-56 (SEQ ID NOS:3-25 and 33-35, respectively), as depicted in Figure 4 (a portion of SEQ ID NO:2).

27. The method of claim 25 in which the portion of said sequence consists of amino acid numbers 45-57 (SEQ ID NO:6) as depicted in Figure 4 (a portion of SEQ ID NO:2), in which substitutions by said amino acid or amino acid analog occur at residues 47 and 51 of said portion.

28. The method of claim 25 in which said one or more residues are each substituted by a diaminobutyric acid residue and the side chain amino group of said diaminobutyric acid residue is peptide bonded to a sequence of one or more proline residues.

29. The method of claim 27 in which the residues at positions 47 and 51 of said portion are each substituted by a diaminobutyric acid residue and the side chain amino group of said diaminobutyric acid residue is peptide bonded to a proline residue.

30. A method of treating or preventing a disease or disorder by increasing the production of one or more hematopoietic cell types in a subject in need of such

treatment or prevention comprising administering to the subject an amount of a circularized protein effective to increase the production of one or more hematopoietic cell types, the amino acid sequence of which protein consists of one or more portions of the sequence of β-hCG as depicted in Figure 4 (SEQ ID NO:2) in which a cysteine residue is inserted or substituted for a different amino acid residue in at least one of said one or more portions of said sequence, said one or more portions of said sequence containing a second cysteine residue, and in which a disulfide bond is formed between the inserted or substituted cysteine residue and the second cysteine residue present in said one or more portions of said sequence, and also in which one or more residues in at least one of said one or more portions of said sequence are substituted by an amino acid or amino acid analog having a side chain with an amino or carboxyl group, said amino or carboxyl group forming a peptide bond with a second sequence of one or more amino acids, said protein being active to increase the production of one or more hematopoietic cell types.

31. The method of claim 30 in which the circularized protein has an amino acid sequence which consists of β-hCG amino acid numbers 44-57 (SEQ ID NO:12) as depicted in Figure 3 (a portion of SEQ ID NO:2), with cysteine substituted for valine at position 44, and in which the residues positions 47 and 51 of said portion are each substituted by a diaminobutyric acid residue, and the side chain amino group of said diaminobutyric acid residue is peptide bonded to a proline residue.

32. A method of treating or preventing a disease or disorder by increasing the production of one or more hematopoietic cell types in a subject in need of such treatment or prevention comprising administering to the subject an amount of a purified protein effective to increase the production of one or more hematopoietic cell types, which

protein (a) comprises a portion of the amino acid sequence of β-hCG, a peptide having an amino acid sequence consisting of said portion being active to increase the production of one or more hematopoietic cell types; and (b) lacks β-hCG amino acids contiguous to said portion.

33. The method of claim 32 in which the portion consists of amino acid numbers 41-54, 45-54, 47-53, 45-57, 109-119, 41-53, 42-53, 43-53, 44-53, 44-57, 45-53, 46-53, 45-54, 45-55, 45-56, 45-58, 47-54, 47-57, 47-56, 47-58, 48-145, 58-145, 109-145, 7-40, 46-65, and 48-56 (SEQ ID NOS:3-25 and 33-35, respectively) as depicted in Figure 4 (a portion of SEQ ID NO:2).

34. The method of claim 32 in which the portion consists of amino acid numbers 45-57 (SEQ ID NO:6) as depicted in Figure 4 (a portion of SEQ ID NO:2).

35. The method of claim 32 in which the subject is a human.

36. The method of claim 32 in which the protein is a fusion protein, said fusion protein comprising said portion joined via a peptide bond to a protein sequence of a protein different from β-hCG.

37. The method of claim 32 in which the subject has HIV infection, idiopathic thrombocytopenic purpura, an anemia, or a neutropenia.

38. The method of claim 32 in which the subject has undergone chemotherapy or radiation therapy.

39. A method of treating or preventing a disease or disorder by increasing the production of one or more hematopoietic cell types in a subject in need of such treatment or prevention comprising administering to the

subject an amount of a first composition effective to increase the production of one or more hematopoietic cell types, said first composition comprising one or more first components of a second composition comprising a sample of native hCG or native β-hCG, said first components being separated from other components of the hCG or β-hCG sample, said first components having a pro-hematopoietic activity, and said second composition having a pro-hematopoietic activity, and said native hCG or native β-hCG not being purified to homogeneity in said second composition.

40. The method of claim 39 in which said first components have an approximate apparent molecular weight selected from the group consisting of 40 kD, 15 kD and 3 kD, wherein said apparent molecular weight is determined by elution from a gel filtration sizing column relative to the elution of a native hCG heterodimer, having a molecular weight of 77 kD, and a β-hCG core protein, having a molecular weight of 10 kD.

41. A method of treating or preventing a disease or disorder by increasing the production of one or more hematopoietic cell types in a subject in need of such treatment or prevention comprising administering to the subject an amount of a first composition effective to increase the production of one or more hematopoietic cell types, said first composition being produced by a process comprising the following steps:

(a) subjecting a second composition comprising native hCG or native β-hCG, said second composition having a pro-hematopoietic activity, said native hCG or native β-hCG not being purified to homogeneity in said second composition, to a size fractionation procedure; and

(b) recovering fractions having a pro-hematopoietic activity.

42. The method of claim 41, in which the recovered fractions contain material having an approximate apparent molecular weight selected from the group consisting of 40 kD, 15 kD and 3 kD, wherein said apparent molecular weight is determined by elution from a gel filtration sizing column relative to the elution of a native hCG heterodimer, having a molecular weight of 77 kD, and a β-hCG core protein, having a molecular weight of 10 kD.

43. The method of claim 39 or 41 in which the subject is a human.

44. The method of claim 39 or 41 in which the subject is a human.

45. The method of claim 39 or 41 in which the subject has HIV infection, idiopathic thrombocytopenic purpura, an anemia, or a neutropenia.

46. The method of claim 39 or 41 in which the subject has undergone chemotherapy or radiation therapy.

47. A method of treating or preventing a disease or disorder by increasing the numbers of one or more hematopoietic cell types in a subject in need of such treatment or prevention comprising:

(a) contacting *in vitro* a non-terminally differentiated hematopoietic cell with a composition comprising an amount of a preparation comprising hCG or β-hCG, effective to increase proliferation of the cell, under conditions suitable and for a time period sufficient to increase the numbers of said hematopoietic cell; and

(b) administering at least a portion of the hematopoietic cells resulting from step (a), or cells produced therefrom, to the subject.

48. The method of claim 47 in which the non-terminally differentiated cell is a hematopoietic stem or progenitor cell.

49. The method of claim 48 in which the non-terminally differentiated hematopoietic cell is from bone marrow or from blood.

50. The method of claim 49 in which the non-terminally differentiated hematopoietic cell is from autologous bone marrow.

51. The method of claim 47 in which the subject has HIV infection, idiopathic thrombocytopenic purpura, an anemia or a neutropenia.

52. The method of claim 47 in which the subject has undergone chemotherapy or radiation therapy.

53. The method of claim 47 in which the cell contains a recombinant nucleic acid such that it is inheritable and capable of expression by progeny of said cell.

54. A method of treating or preventing a disease or disorder by increasing the numbers of one or more hematopoietic cell types in a subject in need of such treatment or prevention comprising:

(a) contacting *in vitro* a non-terminally differentiated hematopoietic cell with a composition comprising an amount of a purified protein effective to increase proliferation of the cell, under conditions suitable and for a time period sufficient to increase the numbers of said hematopoietic cell, which protein comprises one or more portions of the amino acid sequence of β-hCG, a peptide having an amino acid sequence consisting of said one or more portions being

active to increase the production of one or more hematopoietic cell types; and

(b) administering at least a portion of the hematopoietic cells resulting from step (a) or cells produced therefrom, to the subject.

55. The method of claim 54 in which the amino acid sequence of at least one of said one or more portions is selected from the group consisting of amino acid numbers 41-54, 45-54, 47-53, 45-57, 109-119, 41-53, 42-53, 43-53, 44-53, 44-57, 45-53, 46-53, 45-54, 45-55, 45-56, 45-58, 47-54, 47-57, 47-56, 47-58, 48-145, 58-145, 109-145, 7-40, 46-65, and 48-56 (SEQ ID NOS:3-25 and 33-35, respectively), as depicted in Figure 4 (a portion of SEQ ID NO:2).

56. The method of claim 54 in which the amino acid sequence consists of amino acid numbers 45-57 (SEQ ID NO:6) as depicted in Figure 4 (a portion of SEQ ID NO:2).

57. The method of claim 54 in which the protein is N-acetylated and has a C-terminal amide.

58. The method of claim 54 in which the purified protein comprises two or more at least five amino acid, non-naturally contiguous portions of the amino acid sequence of β-hCG as depicted in Figure 4 (SEQ ID NO:2), in which said portions are linked via a peptide bond between the N-terminus of a first said portion and the C-terminus of a second said portion.

59. The method of claim 58, in which the amino acid sequence of said protein is selected from the group consisting of β-hCG amino acids 45-57 (SEQ ID NO:6) linked at the C-terminus via a peptide bond to the N-terminus of β-hCG amino acids 109-119 (SEQ ID NO:7), β-hCG amino acids 110-119 (SEQ ID NO:27) linked at the C-terminus via a peptide bond to the N-terminus of β-hCG amino acids 45-57 (SEQ ID NO:6), and

β-hCG amino acids 47-57 (SEQ ID NO:28) linked at the C-terminus via a peptide bond to the N-terminus of β-hCG amino acids 108-119 (SEQ ID NO:29) as depicted in Figure 4 (SEQ ID NO:2).

60. The method of claim 54 in which the non-terminally differentiated cell is a hematopoietic stem or progenitor cell.

61. The method of claim 54 in which the non-terminally differentiated hematopoietic cell is from bone marrow or blood.

62. The method of claim 61 in which the non-terminally differentiated hematopoietic cell is from autologous bone marrow.

63. The method of claim 54 in which the subject has HIV infection, idiopathic thrombocytopenic purpura, an anemia or a neutropenia.

64. The method of claim 54 in which the subject has undergone chemotherapy or radiation therapy.

65. The method of claim 54 in which the cell contains a recombinant nucleic acid such that it is inheritable and capable of expression by progeny of said cell.

66. A method of treating or preventing a disease or disorder by increasing the numbers of one or more hematopoietic cell types in a subject in need of such treatment or prevention comprising:

(a) contacting *in vitro* a non-terminally differentiated hematopoietic cell with a composition comprising an amount of a purified derivative of a protein, said derivative being effective to increase proliferation of the cell, under conditions suitable and

for a time period sufficient to increase the numbers of said hematopoietic cell, which protein comprises one or more portions of the amino acid sequence of β-hCG, a peptide having an amino acid sequence consisting of said one or more portions being active to increase the production of one or more hematopoietic cell types; and

(b) administering at least a portion of the hematopoietic cells resulting from step (a), or cells produced therefrom, to the subject.

67. The method of claim 66 in which the amino acid sequence of at least one of said one or more portions is selected from the group consisting of amino acid numbers 41-54, 45-54, 47-53, 45-57, 109-119, 41-53, 42-53, 43-53, 44-53, 44-57, 45-53, 46-53, 45-54, 45-55, 45-56, 45-58, 47-54, 47-57, 47-56, 47-58, 48-145, 58-145, 109-145, 7-40, 46-65, and 48-56 (SEQ ID NOS:3-25 and 33-35, respectively) as depicted in Figure 4 (a portion of SEQ ID NO:2).

68. The method of claim 66 in which the purified derivative comprises two or more at least five amino acid, non-naturally contiguous portions of the amino acid sequence of β-hCG as depicted in Figure 4 (SEQ ID NO:2), in which said portions are linked via a peptide bond between the N-terminus of a first said portion and the C-terminus of a second said portion.

69. The method of claim 68, in which the amino acid sequence of said derivative is selected from the group consisting of β-hCG amino acids 45-57 (SEQ ID NO:6) linked at the C-terminus via a peptide bond to the N-terminus of β-hCG amino acids 109-119 (SEQ ID NO:7), β-hCG amino acids 110-119 (SEQ ID NO:27) linked at the C-terminus via a peptide bond to the N-terminus of β-hCG amino acids 45-57 (SEQ ID NO:6), and β-hCG amino acids 47-57 (SEQ ID NO:28) linked at the C-terminus via a peptide bond to the N-terminus of β-hCG amino

acids 108-119 (SEQ ID NO:29) as depicted in Figure 4 (SEQ ID NO:2).

70. The method of claim 66 in which the derivative has one or more D-amino acids or non-classical amino acids.

71. The method of claim 66 in which the non-terminally differentiated cell is a hematopoietic stem or progenitor cell.

72. The method of claim 66 in which the non-terminally differentiated hematopoietic cell is from bone marrow or from blood.

73. The method of claim 72 in which the non-terminally differentiated hematopoietic cell is from autologous bone marrow.

74. The method of claim 66 in which the subject has HIV infection, idiopathic thrombocytopenic purpura, an anemia, or a neutropenia.

75. The method of claim 66 in which the subject has undergone chemotherapy or radiation therapy.

76. The method of claim 66 in which the cell contains a recombinant nucleic acid such that it is inheritable and capable of expression by progeny of said cell.

77. A method of treating or preventing a disease or disorder by increasing the numbers of one or more hematopoietic cell types in a subject in need of such treatment or prevention comprising:

(a) contacting *in vitro* a non-terminally differentiated hematopoietic cell with a composition comprising an amount of a circularized protein effective to increase proliferation of the cell, under

conditions suitable and for a time period sufficient to increase the numbers of said hematopoietic cell, the amino acid sequence of which protein consists of one or more portions of the sequence of β-hCG as depicted in Figure 4 (SEQ ID NO:2) in which a cysteine residue is inserted or substituted for a different amino acid residue in at least one of said one or more portions of said sequence, said one or more portions of said sequence containing a second cysteine residue, and in which a disulfide bond is formed between the inserted or substituted cysteine residue and the second cysteine residue present in said one or more portions of said sequence, said protein being active to increase the production of one or more hematopoietic cell types; and

(b) administering at least a portion of the hematopoietic cells resulting from step (a), or cells produced therefrom, to the subject.

78. The method of claim 77 in which at least one of said one or more portions has an amino acid sequence selected from the group consisting of amino acid numbers 41-54, 45-54, 47-53, 45-57, 109-119, 41-53, 42-53, 43-53, 44-53, 44-57, 45-53, 46-53, 45-54, 45-55, 45-56, 45-58, 47-54, 47-57, 47-56, 47-58, 48-145, 58-145, 109-145, 7-40, 46-65, and 48-56 (SEQ ID NOS:3-25 and 33-35, respectively) as depicted in Figure 4 (a portion of SEQ ID NO:2).

79. The method of claim 78 in which the circularized protein has an amino acid sequence which consists of β-hCG amino acid numbers 44-57 (SEQ ID NO:12) as depicted in Figure 4 (a portion of SEQ ID NO:2), with cysteine substituted for valine at position 44.

80. The method of claim 77 in which said circularized protein consists of two or more at least five amino acid, non-naturally contiguous portions of the sequence of β-hCG as depicted in Figure 4 (SEQ ID NO:2), in which the portions are

linked via a peptide bond between the N-terminus of a first said portion and the C-terminus of a second said portion.

81. The method of claim 80, in which the first portion consists of amino acid numbers 45-57 (SEQ ID NO:6) and the second portion consists of amino acid numbers 110-119 (SEQ ID NO:27) as depicted in Figure 4 (SEQ ID NO:2); and in which a disulfide bond is formed between the cysteine residues at a amino acids 57 and 110 of said portions.

82. A method of treating or preventing a disease or disorder by increasing the numbers of one or more hematopoietic cell types in a subject in need of such treatment or prevention comprising:

(a) contacting *in vitro* a non-terminally differentiated hematopoietic cell with a composition comprising an amount of a purified protein effective to increase proliferation of the cell, under conditions suitable and for a time period sufficient to increase the numbers of said hematopoietic cell, the amino acid sequence of which protein consists of one or more portions of the sequence of β-hCG as depicted in Figure 4 (SEQ ID NO:2) in which one or more residues in at least one of said one or more portions of said sequence are substituted by an amino acid or amino acid analog having a side chain with an amino or carboxyl group, said amino or carboxyl group forming a peptide bond with a second sequence of one or more amino acids, said protein being active to increase the production of one or more hematopoietic cell types; and

(b) administering at least a portion of the hematopoietic cells resulting from step (a), or cells produced therefrom, to the subject.

83. The method of claim 82 in which said at least one portion of said sequence has an amino acid sequence selected from the group consisting of amino acid numbers 41-54, 45-54,

47-53, 45-57, 109-119, 41-53, 42-53, 43-53, 44-53, 44-57, 45-53, 46-53, 45-54, 45-55, 45-56, 45-58, 47-54, 47-57, 47-56, 47-58, 48-145, 58-145, 109-145, 7-40, 46-65, and 48-56 (SEQ ID NOS:3-25 and 33-35, respectively) as depicted in Figure 4 (a portion of SEQ ID NO:2).

84. The method of claim 82 in which the portion of said sequence consists of amino acid numbers 45-57 (SEQ ID NO:6) as depicted in Figure 4 (a portion of SEQ ID NO:2), and in which substitutions by said amino acid or amino acid analog occur at residues 47 and 51 of said portion.

85. The method of claim 82 in which said one or more residues are each substituted by a diaminobutyric acid residue and the side chain amino group of said diaminobutyric acid residue is peptide bonded to a sequence of one or more proline residues.

86. The method of claim 84 in which the residues at positions 47 and 51 of said portion are each substituted by a diaminobutyric acid residue and the side chain amino group of said diaminobutyric acid residue is peptide bonded to a proline residue.

87. A method of treating or preventing a disease or disorder by increasing the numbers of one or more hematopoietic cell types in a subject in need of such treatment or prevention comprising

(a) contacting *in vitro* a non-terminally differentiated hematopoietic cell with a composition comprising an amount of a circularized protein effective to increase proliferation of the cell, under conditions suitable and for a time period sufficient to increase the numbers of said hematopoietic cell, the amino acid sequence of which protein consists of one or more portions of the sequence of β-hCG as depicted in Figure 4 (SEQ ID NO:2) in which a cysteine residue is

inserted or substituted for a different amino acid residue in at least one of said one or more portions of said sequence, said one or more portions of said sequence containing a second cysteine residue, and in which a disulfide bond is formed between the inserted or substituted cysteine residue and the second cysteine residue present said one or more portions of said sequence, and also in which one or more residues in at least one of said one or more portions of said sequence are substituted by an amino acid or amino acid analog having a side chain with an amino or carboxyl group, said amino or carboxyl group forming a peptide bond with a second sequence of one or more amino acids, said protein being active to increase the production of one or more hematopoietic cell types; and

(b) administering at least a portion of the hematopoietic cells resulting from step (a), or cells produced therefrom, to the subject.

88. The method of claim 87 in which the circularized protein has an amino acid sequence which consists of β-hCG amino acid numbers 44-57 (SEQ ID NO:12) as depicted in Figure 3 (a portion of SEQ ID NO:2), with cysteine substituted for valine at position 44, and in which the residues at positions 47 and 51 of said portion are each substituted by a diaminobutyric acid residue and the side chain amino group of said diaminobutyric acid residue is peptide bonded to a proline residue.

89. A method of treating or preventing a disease or disorder by increasing the numbers of one or more hematopoietic cell types in a subject in need of such treatment or prevention comprising

(a) contacting *in vitro* a non-terminally differentiated hematopoietic cell with a composition comprising an amount of a purified protein effective to increase proliferation of the cell, under conditions

suitable and for a time period sufficient to increase the numbers of said hematopoietic cell, which protein

(i) comprises a portion of the amino acid sequence of β-hCG, a peptide having an amino acid sequence consisting of said portion being active to increase the production of one or more hematopoietic cell types; and

(ii) lacks β-hCG amino acids contiguous to said portion; and

(b) administering at least a portion of the resulting hematopoietic cells, or cells produced therefrom, to the subject.

90. The method of claim 89 in which the portion has an amino acid sequence selected from the group consisting of amino acid numbers 41-54, 45-54, 47-53, 45-57, 109-119, 41-53, 42-53, 43-53, 44-53, 44-57, 45-53, 46-53, 45-54, 45-55, 45-56, 45-58, 47-54, 47-57, 47-56, 47-58, 48-145, 58-145, 109-145, 7-40, 46-65, and 48-56 (SEQ ID NOS:3-25 and 33-35, respectively) as depicted in Figure 4 (a portion of SEQ ID NO:2).

91. The method of claim 90 in which the portion consists of amino acid numbers 45-57 (SEQ ID NO:6) as depicted in Figure 4 (a portion of SEQ ID NO:2).

92. The method of claim 89 in which the protein is a fusion protein, said fusion protein comprising the portion joined via a peptide bond to a protein sequence of a protein different from β-hCG.

93. The method of claim 89 in which the non-terminally differentiated hematopoietic cell is from bone marrow or blood.

94. The method of claim 93 in which the non-terminally differentiated hematopoietic cell is from autologous bone marrow.

95. The method of claim 89 in which the non-terminally differentiated hematopoietic cell is from the subject, and the subject is a human.

96. The method of claim 89 in which the subject has HIV infection, idiopathic thrombocytopenic purpura, an anemia, or a neutropenia.

97. The method of claim 89 in which the subject has undergone chemotherapy or radiation therapy.

98. The method of claim 89 in which the cell contains a recombinant nucleic acid such that it is inheritable and capable of expression by progeny of said cell.

99. A method of treating or preventing a disease or disorder by increasing the numbers of one or more hematopoietic cell types in a subject in need of such treatment or prevention comprising:

(a) contacting *in vitro* a non-terminally differentiated hematopoietic cell with a composition comprising an amount of a first composition effective to increase proliferation of the cell, under conditions suitable and for a time period sufficient to increase the numbers of said hematopoietic cell, which first composition comprises one or more first components of a second composition comprising a sample of native hCG or native β-hCG, said first components being separated from other components of the hCG or β-hCG sample, said first components having a pro-hematopoietic activity, and said second composition having a pro-hematopoietic activity, and said native hCG or native β-hCG not being purified to homogeneity in said second composition; and

(b) administering at least a portion of the resulting hematopoietic cells, or cells produced therefrom, to the subject.

100. The method of claim 99 in which said first components have an approximate apparent molecular weight selected from the group consisting of 40 kD, 15 kD and 3 kD, wherein said apparent molecular weight is determined by elution from a gel filtration sizing column relative to the elution of a native hCG heterodimer, having a molecular weight of 77 kD, and a β-hCG core protein, having a molecular weight of 10 kD.

101. A method of treating or preventing a disease or disorder by increasing the numbers of one or more hematopoietic cell types in a subject in need of such treatment or prevention comprising

(a) contacting *in vitro* a non-terminally differentiated hematopoietic cell with a composition comprising an amount of a first composition effective to increase proliferation of the cell, under conditions suitable and for a time period sufficient to increase the numbers of said hematopoietic cell, said first composition being produced by a process comprising the following steps:

(i) subjecting a second composition comprising native hCG or native β-hCG, said second composition having a pro-hematopoietic activity, said native hCG or native β-hCG not being purified to homogeneity in said second composition, to a size fractionation procedure; and

(ii) recovering fractions having a pro-hematopoietic activity; and

(b) administering at least a portion of the resulting hematopoietic cells, or cells produced therefrom, to the subject.

102. The method of claim 101, in which the recovered fractions contain material having an approximate apparent molecular weight selected from the group consisting of 40 kD, 15 kD and 3 kD, wherein said apparent molecular weight is determined by elution from a gel filtration sizing column relative to the elution of a native hCG heterodimer, having a molecular weight of 77 kD, and a β-hCG core protein, having a molecular weight of 10 kD.

103. The method of claim 99 or 101 in which the non-terminally differentiated hematopoietic cell is from bone marrow or blood.

104. The method of claim 103 in which the non-terminally differentiated hematopoietic cell is from autologous bone marrow.

105. The method of claim 99 or 101 in which the non-terminally differentiated hematopoietic cell is from the subject, and the subject is a human.

106. The method of claim 99 or 101 in which the subject has HIV infection, idiopathic thrombocytopenic purpura, an anemia, or a neutropenia.

107. The method of claim 99 or 101 in which the subject has undergone chemotherapy or radiation therapy.

108. The method of claim 99 or 101 in which the cell contains a recombinant nucleic acid such that it is inheritable and capable of expression by progeny of said cell.

109. A method for screening a preparation comprising hCG or an hCG α chain or hCG β chain or a derivative of hCG or of said alpha or beta chain or a fraction of a source of native hCG or native β-hCG, for pro-hematopoietic activity

comprising assaying said preparation for the ability to induce an increase in hematopoietic cell numbers.

110. The method of claim 109 in which the preparation is screened by a method comprising measuring the number of colonies formed from hematopoietic stem or progenitor cells, which cells have been contacted with the preparation; and comparing the number of colonies formed from the cells contacted with the preparation with the number of colonies formed from cells not so contacted with the preparation, wherein a higher number of colonies formed from said contacted cells indicates that the preparation has pro-hematopoietic activity.

111. The method of claim 109 in which the preparation is screened by a method comprising measuring the number of $CD4^+$ T cells in an SIV infected monkey, which monkey has been exposed to the preparation; and comparing the number of $CD4^+$ T cells in the monkey which has been exposed to the preparation with the number of $CD4^+$ T cells in a monkey not so exposed, wherein a higher number of $CD4^+$ T cells in said exposed monkey indicates that the preparation has pro-hematopoietic activity.

112. A pharmaceutical composition comprising an amount of a preparation comprising hCG or β-hCG effective for treating an hematopoietic deficiency; and a pharmaceutically acceptable carrier.

113. The pharmaceutical composition of claim 112 which is formulated as a controlled release formulation.

114. A pharmaceutical composition comprising an amount effective for treatment of an hematopoietic deficiency of a purified protein, the amino acid sequence of which protein is selected from the group consisting of amino acid numbers 41-54, 45-54, 47-53, 45-57, 109-119, 41-53, 42-53, 43-53, 44-53,

44-57, 45-53, 46-53, 45-54, 45-55, 45-56, 45-58, 47-54, 47-57, 47-56, 47-58, 48-145, 58-145, 109-145, 7-40, 46-65, and 48-56 (SEQ ID NOS:3-25 and 33-35, respectively) as depicted in Figure 4 (a portion of SEQ ID NO:2); and a pharmaceutically acceptable carrier.

115. The pharmaceutical composition of claim 114 in which the amino acid sequence of the protein consists of amino acid numbers 45-57 (SEQ ID NO:6) as depicted in Figure 4 (a portion of SEQ ID NO:2).

116. A pharmaceutical composition comprising a therapeutically effective amount of a purified protein effective for treatment of hematopoietic deficiency, the amino acid sequence of which comprises two or more at least five amino acid, non-naturally contiguous portions of the amino acid sequence of β-hCG as depicted in Figure 4 (SEQ ID NO:2), in which the portions are linked via a peptide bond between the N-terminus of a first said portion and the C-terminus of a said second portion; and a pharmaceutically acceptable carrier.

117. The pharmaceutical composition of claim 116, in which the amino acid sequence of said protein is selected from the group consisting of β-hCG amino acids 45-57 (SEQ ID NO:6) linked at the C-terminus via a peptide bond to the N-terminus of β-hCG amino acids 109-119 (SEQ ID NO:7), β-hCG amino acids 110-119 (SEQ ID NO:27) linked at the C-terminus via a peptide bond to the N-terminus of β-hCG amino acids 45-57 (SEQ ID NO:6), and β-hCG amino acids 47-57 (SEQ ID NO:28) linked at the C-terminus via a peptide bond to the N-terminus of β-hCG amino acids 108-119 (SEQ ID NO:29) as depicted in Figure 4 (SEQ ID NO:2).

118. The pharmaceutical composition of claim 116 in which the protein is a fusion protein, said fusion protein comprising at least one of said portions of the β-hCG amino

acid sequence joined via a peptide bond to a sequence of a protein different from β-hCG.

119. The pharmaceutical composition of claim 114 or 116 in which the protein is N-acetylated and has a C-terminal amide.

120. The pharmaceutical composition of claim 114 or 116 which is formulated as a controlled release formulation.

121. A pharmaceutical composition comprising an amount effective for treatment of an hematopoietic deficiency of a derivative of a protein, the amino acid sequence of which protein is selected from the group consisting of amino acid numbers 41-54, 45-54, 47-53, 45-57, 109-119, 41-53, 42-53, 43-53, 44-53, 44-57, 45-53, 46-53, 45-54, 45-55, 45-56, 45-58, 47-54, 47-57, 47-56, 47-58, 48-145, 58-145, 109-145, 7-40, 46-65, and 48-56 (SEQ ID NOS:3-25 and 33-35, respectively) as depicted in Figure 4 (a portion of SEQ ID NO:2), said derivative being effective for treatment of hematopoietic deficiency; and a pharmaceutically acceptable carrier.

122. The pharmaceutical composition of claim 121 in which the amino acid sequence of the protein consists of amino acid numbers 45-57 (SEQ ID NO:6) as depicted in Figure 4 (a portion of SEQ ID NO:2).

123. A pharmaceutical composition comprising a therapeutically effective amount of a derivative of a protein, the amino acid sequence of which protein comprises two or more at least five amino acid, non-naturally contiguous portions of the amino acid sequence of β-hCG as depicted in Figure 4 (SEQ ID NO:2), in which the portions are linked via a peptide bond between the N-terminus of a first said portion and the C-terminus of a second said portion, said derivative being effective for treatment of

hematopoietic deficiency; and a pharmaceutically acceptable carrier.

124. The pharmaceutical composition of claim 123, in which the amino acid sequence of said derivative is selected from the group consisting of β-hCG amino acids 45-57 (SEQ ID NO:6) linked at the C-terminus via a peptide bond to the N-terminus of β-hCG amino acids 109-119 (SEQ ID NO:7), β-hCG amino acids 110-119 (SEQ ID NO:27) linked at the C-terminus via a peptide bond to the N-terminus of β-hCG amino acids 45-57 (SEQ ID NO:6), and β-hCG amino acids 47-57 (SEQ ID NO:28) linked at the C-terminus via a peptide bond to the N-terminus of β-hCG amino acids 108-119 (SEQ ID NO:29) as depicted in Figure 4 (SEQ ID NO:2).

125. The pharmaceutical composition of claim 121 or 123 in which the derivative contains one or more D-amino acids or one or more non-classical amino acids.

126. A pharmaceutical composition comprising an amount effective for treatment of an hematopoietic deficiency of a circularized protein, the amino acid sequence of which protein consists of one or more portions of the sequence of β-hCG as depicted in Figure 4 (SEQ ID NO:2) in which a cysteine residue is inserted or substituted for a different amino acid residue in at least one of said one or more portions of said sequence, said one or more portions of said sequence containing a second cysteine residue, and in which a disulfide bond is formed between the inserted or substituted cysteine residue and the second cysteine residue present in said one or more portions of said sequence, said protein being active for treatment of an hematopoietic deficiency; and a pharmaceutically acceptable carrier.

127. The pharmaceutical composition of claim 126 in which the circularized protein has an amino acid sequence selected from the group consisting of amino acid numbers 41-

54, 45-54, 47-53, 45-57, 109-119, 41-53, 42-53, 43-53, 44-53, 44-57, 45-53, 46-53, 45-54, 45-55, 45-56, 45-58, 47-54, 47-57, 47-56, 47-58, 48-145, 58-145, 109-145, 7-40, 46-65, and 48-56 (SEQ ID NOS:3-25 and 33-35, respectively) as depicted in Figure 4 (a portion of SEQ ID NO:2).

128. The pharmaceutical composition of claim 127 in which the circularized protein has an amino acid sequence which consists of β-hCG amino acid numbers 44-57 (SEQ ID NO:12) as depicted in Figure 4 (a portion of SEQ ID NO:2), with cysteine substituted for valine at position 44.

129. The pharmaceutical composition of claim 126, in which the amino acid sequence of said circularized protein consists of two or more at least five amino acid, non-naturally contiguous portions of the β-hCG sequence as depicted in Figure 4 (SEQ ID NO:2) in which the portions are linked via a peptide bond between the N-terminus of a first said portion and the C-terminus of a second said portion.

130. The pharmaceutical composition of claim 129, in which the first portion consists of amino acid numbers 45-57 (SEQ ID NO:6) and the second portion consists of amino acid numbers 110-119 (SEQ ID NO:27) as depicted in Figure 4 (SEQ ID NO:2); and in which a disulfide bond is formed between the cysteine residue at amino acids 57 and 110 of said portions.

131. A pharmaceutical composition comprising an amount effective for treatment of an hematopoietic deficiency of a purified protein, the amino acid sequence of which consists of one or more portions of the sequence of β-hCG as depicted in Figure 4 (SEQ ID NO:2) in which one or more residues in at least one of said one or more portions of said sequence are substituted by an amino acid or amino acid analog having a side chain with an amino or carboxyl group, said amino or carboxyl group forming a peptide bond with a second sequence of one or more amino acids, said protein being active for

treatment of an hematopoietic deficiency; and a pharmaceutically acceptable carrier.

132. The pharmaceutical composition of claim 131, in which said at least one portion of said sequence has an amino acid sequence selected from the group consisting of amino acid numbers 41-54, 45-54, 47-53, 45-57, 109-119, 41-53, 42-53, 43-53, 44-53, 44-57, 45-53, 46-53, 45-54, 45-55, 45-56, 45-58, 47-54, 47-57, 47-56, 47-58, 48-145, 58-145, 109-145, 7-40, 46-65, and 48-56 (SEQ ID NOS:3-25 and 33-35, respectively) as depicted in Figure 4 (a portion of SEQ ID NO:2).

133. The pharmaceutical composition of claim 131 in which said portion of said sequence consists of amino acid numbers 45-57 (SEQ ID NO:6) as depicted in Figure 4 (a portion of SEQ ID NO:2), and in which substitutions by said amino acid or amino acid analog occur at residues 47 and 51 of said portion.

134. The pharmaceutical composition of claim 131 in which said one or more residues are each substituted by a diaminobutyric acid residue and the side chain amino group of said diaminobutyric acid residue is peptide bonded to a sequence of one or more proline residues.

135. The pharmaceutical composition of claim 133 in which the residues at positions 47 and 51 of said portion are each substituted by a diaminobutyric acid residue and the side chain amino group of said diaminobutyric acid residue is peptide bonded to a proline residue.

136. A pharmaceutical composition comprising an amount effective for treatment of an hematopoietic deficiency of a circularized protein, the amino acid sequence of which consists of one or more portions of the sequence of β-hCG as depicted in Figure 4 (SEQ ID NO:2) in which a cysteine

residue is inserted or substituted for a different amino acid residue in at least one of said one or more portions of said sequence, said one or more portions of said sequence containing a second cysteine residue, and in which a disulfide bond is formed between the inserted or substituted cysteine residue and the second cysteine residue present in said one or more portions of said sequence, and also in which one or more residues in at least one of said one or more portions of said sequence are substituted by an amino acid or amino acid analog having a side chain with an amino or carboxyl group, said amino or carboxyl group forming a peptide bond with a second sequence of one or more amino acids, said protein being active for treatment of an hematopoietic deficiency; and a pharmaceutically acceptable carrier.

137. The pharmaceutical composition of claim 136 in which said protein consists β-hCG amino acid numbers 44-57 (SEQ ID NO:12) as depicted in Figure 4 (a portion of SEQ ID NO:2), and in which cysteine is substituted for valine at position 44, and in which the residues at positions 47 and 51 of said portion are each substituted by a diaminobutyric acid residue and the side chain amino group of said diaminobutyric acid residue is peptide bonded to a proline residue; and a pharmaceutically acceptable carrier.

138. A pharmaceutical composition comprising a therapeutically effective amount of a first composition comprising one or more first components of a second composition comprising a sample of native hCG or native β-hCG, said first components being separated from other components of the hCG or β-hCG sample, said first components having a pro-hematopoietic activity, and said second composition having a pro-hematopoietic activity, and said native hCG or native β-hCG not being purified to homogeneity in said second composition, said first composition being

active for treatment of an hematopoietic deficiency; and a pharmaceutically acceptable carrier.

139. The pharmaceutical composition of claim 138 in which said first components are separated from said other components by sizing column chromatography.

140. The pharmaceutical composition of claim 139 in which said sizing column chromatography is performed using a SUPERDEX™ 200 column.

141. The pharmaceutical composition of claim 139 in which said first components have an approximate apparent molecular weight selected from the group consisting of 40 kD, 15 kD and 3 kD, wherein said apparent molecular weight is determined by elution from a gel filtration sizing column relative to the elution of a native hCG heterodimer, having a molecular weight of 77 kD, and a β-hCG core protein, having a molecular weight of 10 kD.

142. A pharmceutical composition comprising a therapeutically effective amount of a first composition, said first composition produced by a process comprising the following steps:

(a) subjecting a second composition comprising native hCG or native β-hCG, said second composition having a pro-hematopoietic activity, said native hCG or β-hCG not being purified to homogeneity in said second composition, to a size fractionation procedure; and

(b) recovering fractions having a pro-hematopoietic activity,

said first composition being active for treatment of an hematopoietic deficiency; and a pharmaceutically acceptable carrier.

143. The pharmaceutical composition of claim 142, in which the recovered fractions contain material having an

approximate apparent molecular weight selected from the group consisting of 40 kD, 15 kD and 3 kD, wherein said apparent molecular weight is determined by elution from a gel filtration sizing column relative to the elution of a native hCG heterodimer, having a molecular weight of 77 kD, and a β-hCG core protein, having a molecular weight of 10 kD.

144. The pharmaceutical composition of claim 142, in which the sample of hCG is early pregnancy urine.

145. A method of increasing the amount of hematopoietic cells comprising contacting *in vitro* a non-terminally differentiated hematopoietic cell with a composition comprising an amount of a preparation comprising hCG or β-hCG effective to increase proliferation of the cell, under conditions suitable and for a time period sufficient to increase the numbers of said hematopoietic cell.

146. A method of increasing the amount of hematopoietic cells comprising contacting *in vitro* a non-terminally differentiated hematopoietic cell with a composition comprising an amount of a purified protein effective to increase proliferation of the cell, under conditions suitable and for a time period sufficient to increase the numbers of said hematopoietic cell, which protein contains one or more portions of the amino acid sequence of β-hCG, a peptide having an amino acid sequence consisting of said one or more portions being active to increase proliferation of the cell.

147. The method of claim 146 in which the amino acid sequence of at least one of said one or more portions is selected from the group consisting of amino acid numbers 41-54, 45-54, 47-53, 45-57, 109-119, 41-53, 42-53, 43-53, 44-53, 44-57, 45-53, 46-53, 45-54, 45-55, 45-56, 45-58, 47-54, 47-57, 47-56, 47-58, 48-145, 58-145, 109-145, 7-40, 46-65, and 48-56 (SEQ ID NOS:3-25 and 33-35, respectively) as depicted in Figure 4 (a portion of SEQ ID NO:2).

148. The method of claim 147 in which the amino acid sequence consists of amino acid numbers 45-57 (SEQ ID NO:6) as depicted in Figure 4 (a portion of SEQ ID NO:2).

149. The method of claim 146 in which the purified protein comprises two or more at least five amino acid, non-naturally contiguous portions of the amino acid sequence of β-hCG as depicted in Figure 4 (SEQ ID NO:2), in which said portions are linked via a peptide bond between the N-terminus of a first said portion and the C-terminus of a second said portion.

150. The method of claim 149, in which the amino acid sequence of said protein is selected from the group consisting of β-hCG amino acids 45-57 (SEQ ID NO:6) linked at the C-terminus via a peptide bond to the N-terminus of β-hCG amino acids 109-119 (SEQ ID NO:7), β-hCG amino acids 110-119 (SEQ ID NO:27) linked at the C-terminus via a peptide bond to the N-terminus of β-hCG amino acids 45-57 (SEQ ID NO:6), and β-hCG amino acids 47-57 (SEQ ID NO:28) linked at the C-terminus via a peptide bond to the N-terminus of β-hCG amino acids 108-119 (SEQ ID NO:29) as depicted in Figure 4 (SEQ ID NO:2).

151. The method of claim 146 in which the protein is N-acetylated and has a C-terminal amide.

152. A method of increasing the amount of hematopoietic cells comprising contacting *in vitro* a non-terminally differentiated hematopoietic cell with a composition comprising an amount of a purified derivative of a protein effective to increase proliferation of the cell, under conditions suitable and for a time period sufficient to increase the numbers of said hematopoietic cell, which protein comprises one or more portions of the amino acid sequence of β-hCG, a peptide having an amino acid sequence of

said one or more portions being active to increase proliferation of the cell.

153. The method of claim 152 in which the amino acid sequence of at least one of said one or more portions is selected from the group consisting of amino acid numbers 41-54, 45-54, 47-53, 45-57, 109-119, 41-53, 42-53, 43-53, 44-53, 44-57, 45-53, 46-53, 45-54, 45-55, 45-56, 45-58, 47-54, 47-57, 47-56, 47-58, 48-145, 58-145, 109-145, 7-40, 46-65, and 48-56 (SEQ ID NOS:3-25 and 33-35, respectively) as depicted in Figure 4 (a portion of SEQ ID NO:2).

154. The method of claim 152 in which the purified derivative comprises two or more at least five amino acid, non-naturally contiguous portions of the amino acid sequence of β-hCG as depicted in Figure 4 (SEQ ID NO:2), in which said portions are linked via a peptide bond between the N-terminus of a first said portion and the C-terminus of a second said portion.

155. The method of claim 154, in which the amino acid sequence of said derivative is selected from the group consisting of β-hCG amino acids 45-57 (SEQ ID NO:6) linked at the C-terminus via a peptide bond to the N-terminus of β-hCG amino acids 109-119 (SEQ ID NO:7), β-hCG amino acids 110-119 (SEQ ID NO:27) linked at the C-terminus via a peptide bond to the N-terminus of β-hCG amino acids 45-57 (SEQ ID NO:6), and β-hCG amino acids 47-57 (SEQ ID NO:28) linked at the C-terminus via a peptide bond to the N-terminus of β-hCG amino acids 108-119 (SEQ ID NO:29) as depicted in Figure 4 (SEQ ID NO:2).

156. The method of claim 152 in which the derivative contains one or more D-amino acids or one or more non classical amino acids.

157. A method of increasing the amount of hematopoietic cells comprising contacting *in vitro* a non-terminally differentiated hematopoietic cell with a composition comprising an amount of a circularized protein effective to increase proliferation of the cell, under conditions suitable and for a time period sufficient to increase the numbers of said hematopoietic cell, which protein consists of one or more portions of the sequence of β-hCG as depicted in Figure 4 (SEQ ID NO:2) in which a cysteine residue is inserted or substituted for a different amino acid residue in at least one of said one or more portions of said sequence,said one or more portions of said sequence containing a second cysteine residue, and in which a disulfide bond is formed between the inserted or substituted cysteine residue and the second cysteine residue present in said one or more portions of said sequence, said protein being active to increase proliferation of the cell.

158. The method of claim 157 in which at least one of said portions has an amino acid sequence selected from the group consisting of amino acid numbers 41-54, 45-54, 47-53, 45-57, 109-119, 41-53, 42-53, 43-53, 44-53, 44-57, 45-53, 46-53, 45-54, 45-55, 45-56, 45-58, 47-54, 47-57, 47-56, 47-58, 48-145, 58-145, 109-145, 7-40, 46-65, and 48-56 (SEQ ID NOS:3-25 and 33-35, respectively) as depicted in Figure 4 (a portion of SEQ ID NO:2).

159. The method of claim 157 in which the circularized protein has an amino acid sequence which consists of β-hCG amino acid numbers 44-57 (SEQ ID NO:12) as depicted in Figure 4 (a portion of SEQ ID NO:2), with cysteine substituted for valine at position 44.

160. The method of claim 157 in which said circularized protein consists of two or more at least five amino acid, non-naturally contiguous portions of the sequence of β-hCG as depicted in Figure 4 (SEQ ID NO:2), in which the portions are

linked via a peptide bond between the N-terminus of a first said portion and the C-terminus of a second said portion.

161. The method of claim 160, in which the first portion consists of amino acid numbers 45-57 (SEQ ID NO:6) and the second portion consists of amino acid numbers 110-119 (SEQ ID NO:27) as depicted in Figure 4 (SEQ ID NO:2); and in which a disulfide bond is formed between the cysteine residues at a amino acids 57 and 110 of said portions.

162. A method of increasing the amount of hematopoietic cells comprising contacting *in vitro* a non-terminally differentiated hematopoietic cell with a composition comprising an amount of a purified protein effective to increase proliferation of the cell, under conditions suitable and for a time period sufficient to increase the numbers of said hematopoietic cell, which protein consists of one or more portions of the sequence of β-hCG as depicted in Figure 4 (SEQ ID NO:2) in which one or more residues in at least one of said one or more portions of said sequence are substituted by an amino acid or amino acid analog having a side chain with an amino or carboxyl group, said amino or carboxyl group forming a peptide bond with a second sequence of one or more amino acids, said protein being active to increase the proliferation of the cell.

163. The method of claim 162 in which said at least one portion has an amino acid sequence selected from the group consisting of amino acid numbers 41-54, 45-54, 47-53, 45-57, 109-119, 41-53, 42-53, 43-53, 44-53, 44-57, 45-53, 46-53, 45-54, 45-55, 45-56, 45-58, 47-54, 47-57, 47-56, 47-58, 48-145, 58-145, 109-145, 7-40, 46-65, and 48-56 (SEQ ID NOS:3-25 and 33-35, respectively) as depicted in Figure 4 (a portion of SEQ ID NO:2).

164. The method of claim 162 in which said protein consists of amino acid numbers 45-57 (SEQ ID NO:6) as

depicted in Figure 4 (a portion of SEQ ID NO:2), and in which substitutions by said amino acid or amino acid analog occur at residues 47 and 51 of said portion.

165. The method of claim 162 in which said one or more residues are each substituted by a diaminobutyric acid residue and the side chain amino group of said diaminobutyric acid residue is peptide bonded to a sequence of one or more proline residues.

166. The method of claim 164 in which the residues at positions 47 and 51 of said portion are each substituted by a diaminobutyric acid residue and the side chain amino group of said diaminobutyric acid residue is peptide bonded to a proline residue.

167. A method of increasing the amount of hematopoietic cells comprising contacting *in vitro* a non-terminally differentiated hematopoietic cell with a composition comprising an amount of a circularized protein effective to increase proliferation of the cell, under conditions suitable and for a time period sufficient to increase the numbers of said hematopoietic cell, which protein consists of one or more portions of the sequence of β-hCG as depicted in Figure 4 (SEQ ID NO:2) in which a cysteine residue is inserted or substituted for a different amino acid residue in at least one of said one or more portions of said sequence, said one or more portions of said sequence containing a second cysteine residue, and in which a disulfide bond is formed between the inserted or substituted cysteine residue and the second cysteine residue present in said one or more portions of said sequence, and also in which one or more residues in at least one of said one or more portions of said sequence are substituted by an amino acid or amino acid analog having a side chain with an amino or carboxyl group, said amino or carboxyl group forming a peptide bond with a second sequence of one or more amino

acids, said protein being active to increase proliferation of the cell.

168. The method of claim 167 in which the circularized protein has an amino acid sequence which consists of β-hCG amino acid numbers 44-57 (SEQ ID NO:12) as depicted in Figure 4 (a portion of SEQ ID NO:2), with cysteine substituted for valine at position 44, and in which the residues at positions 47 and 51 of said portion are each substituted by a diaminobutyric acid residue, and the side chain amino group of said diaminobutyric acid residue is peptide bonded to a proline residue.

169. A method of increasing the amount of hematopoietic cells comprising contacting *in vitro* a non-terminally differentiated hematopoietic cell with a composition comprising an amount of a purified protein effective to increase proliferation of the cell, under conditions suitable and for a time period sufficient to increase the numbers of said hematopoietic cell, which protein (a) comprises a portion of the amino acid sequence of β-hCG, a peptide having an amino acid sequence consisting of said portion being active to increase proliferation of the cell; and (b) lacks β-hCG amino acids contiguous to said portion, said protein being active to increase proliferation of the cell.

170. The method of claim 169 in which the portion has an amino acid sequence selected from the group consisting of amino acid numbers 41-54, 45-54, 47-53, 45-57, 109-119, 41-53, 42-53, 43-53, 44-53, 44-57, 45-53, 46-53, 45-54, 45-55, 45-56, 45-58, 47-54, 47-57, 47-56, 47-58, 48-145, 58-145, 109-145, 7-40, 46-65, and 48-56 (SEQ ID NOS:3-25 and 33-35, respectively) as depicted in Figure 4 (a portion of SEQ ID NO:2).

171. The method of claim 169 in which the portion consists of amino acid numbers 45-57 (SEQ ID NO:6) as depicted in Figure 4 (a portion of SEQ ID NO:2).

172. The method of claim 169 in which the protein is a fusion protein, said fusion protein comprising the portion joined via a peptide bond to a protein sequence of a protein different from β-hCG.

173. The method of claim 169 in which the cell contains a recombinant nucleic acid such that it is inheritable and capable of expression by progeny of said cell.

174. A method of increasing the amount of hematopoietic cells comprising contacting *in vitro* a non-terminally differentiated hematopoietic cell with an amount of a first composition effective to increase proliferation of the cell, under conditions suitable and for a time period sufficient to increase the numbers of said hematopoietic cell, which first composition comprises one or more first components of a second composition comprising a sample of native hCG or native β-hCG, said first components being separated from other components of the hCG or β-hCG sample, said first components having a pro-hematopoietic activity, and said second composition having a pro-hematopoietic activity, and said native hCG or native β-hCG not being purified to homogeneity in said second composition, said first composition being active to increase proliferation of the cell.

175. The method of claim 174 in which in said first composition, said first components are separated from said other components by sizing column chromatography.

176. The method of claim 175 in which said sizing column chromatography is performed using a SUPERDEX™ 200 column.

177. The method of claim 175 in which said first components have an approximate apparent molecular weight selected from the group consisting of 40 kD, 15 kD and 3 kD, wherein said apparent molecular weight is determined by elution from a gel filtration sizing column relative to the elution of a native hCG heterodimer, having a molecular weight of 77 kD, and a β-hCG core protein, having a molecular weight of 10 kD.

178. A method of increasing the amount of hematopoietic cells comprising contacting *in vitro* a non-terminally differentiated hematopoietic cell with an amount of a first composition effective to increase proliferation of the cell, under conditions suitable and for a time period sufficient to increase the numbers of said hematopoietic cell, said first composition being produced by a process comprising the following steps:

(a) subjecting a second composition comprising native hCG or native β-hCG, said second composition having a pro-hematopoietic activity, said native hCG or native β-hCG not being purified to homogeneity in said second composition, to a size fractionation procedure; and

(b) recovering fractions having a pro-hematopoietic activity,

said first composition being active to increase proliferation of the cell.

179. The method of claim 178, in which the recovered fractions contain material having an approximate apparent molecular weight selected from the group consisting of 40 kD, 15 kD and 3 kD, wherein said apparent molecular weight is determined by elution from a gel filtration sizing column relative to the elution of a native hCG heterodimer, having a molecular weight of 77 kD, and a β-hCG core protein, having a molecular weight of 10 kD.

180. A method of treating or preventing a disease or disorder by increasing the production of one or more hematopoietic cell types in a subject in need of such treatment or prevention comprising administering to the subject an amount of a nucleic acid encoding a protein, which protein comprises one or more portions of the amino acid sequence of β-hCG, a peptide having an amino acid consisting of said one or more portions being active to increase the production of one or more hematopoietic cell types.

181. A method of treating or preventing a disease or disorder by increasing the production of one or more hematopoietic cell types in a subject in need of such treatment or prevention comprising administering to the subject an amount of a recombinant cell containing a nucleic acid encoding a protein, which protein comprises one or more portions of the amino acid sequence of β-hCG, a peptide having an amino acid consisting of said one or more portions being active to increase the production of one or more hematopoietic cell types.

AMENDED CLAIMS

[received by the International Bureau on 08 December 1997 (08.12.97); original claims 182-185 added; remaining claims unchanged (4 pages)]

180. A method of treating or preventing a disease or disorder by increasing the production of one or more hematopoietic cell types in a subject in need of such treatment or prevention comprising administering to the subject an amount of a nucleic acid encoding a protein, which protein comprises one or more portions of the amino acid sequence of β-hCG, a peptide having an amino acid consisting of said one or more portions being active to increase the production of one or more hematopoietic cell types.

181. A method of treating or preventing a disease or disorder by increasing the production of one or more hematopoietic cell types in a subject in need of such treatment or prevention comprising administering to the subject an amount of a recombinant cell containing a nucleic acid encoding a protein, which protein comprises one or more portions of the amino acid sequence of β-hCG, a peptide having an amino acid consisting of said one or more portions being active to increase the production of one or more hematopoietic cell types.

182. A method of treating or preventing a disease or disorder by increasing the production of one or more hematopoietic cell types in a subject in need of such treatment or prevention comprising administering to the subject an amount of a composition effective to increase the production of one or more hematopoietic cell types, said composition comprising a component characterized by:

(a) having a molecular weight in the range of 3 to 40 kD:

(b) being present in a crude preparation of native hCG or β-hCG; and

(c) having prohematopoietic activity *in vitro* as measured in an assay comprising:

(i) seeding isolated hematopoietic progenitor cells from normal bone marrow or cord blood cells in methylcellulose;

(ii) contacting said cells with said component; and

(iii) comparing the number of colonies formed after 10 days of culture from the cells contacted with the component with the number of colonies formed from cells not so contacted with the component,

wherein a higher number of colonies formed from said contacted cells indicates that the component has pro-hematopoietic activity.

183. A method of treating or preventing a disease or disorder by increasing the numbers of one or more hematopoietic cell types in a subject in need of such treatment or prevention comprising:

(a) contacting *in vitro* a non-terminally differentiated hematopoietic cell with an amount of a composition effective to increase proliferation of the cell, under conditions suitable and for a time period sufficient to increase the numbers of said hematopoietic cell, said composition comprising a component characterized by:

(i) having a molecular weight in the range of 3 to 40 kD:

(ii) being present in a crude preparation of native hCG or β-hCG; and

(iii) having prohematopoietic activity *in vitro* as measured in an assay comprising:

(A) seeding isolated hematopoietic progenitor cells from normal bone marrow or cord blood cells in methylcellulose;

(B) contacting said cells with said component; and

(C) comparing the number of colonies formed after 10 days of culture from the cells contacted with the component with the number

of colonies formed from cells not so contacted with the component,

wherein a higher number of colonies formed from said contacted cells indicates that the component has pro-hematopoietic activity; and

(b) administering at least a portion of the resulting hematopoietic cells, or cells produced therefrom, to the subject.

184. A pharmaceutical composition comprising a therapeutically effective amount of a component characterized by:

(a) having a molecular weight in the range of 3 to 40 kD:

(b) being present in a crude preparation of native hCG or β-hCG; and

(c) having prohematopoietic activity *in vitro* as measured in an assay comprising:

(i) seeding hematopoietic progenitor cells from normal bone marrow or cord blood cells in methylcellulose;

(ii) contacting said cells with said component; and

(iii) comparing the number of colonies formed after 10 days of culture from the cells contacted with the component with the number of colonies formed from cells not so contacted with the component,

wherein a higher number of colonies formed from said contacted cells indicates that the component has pro-hematopoietic activity;

and a pharmaceutically acceptable carrier.

185. A method of increasing the amount of hematopoietic cells comprising contacting *in vitro* a non-terminally differentiated hematopoietic cell with an amount of a composition effective to increase proliferation of the cell,

under conditions suitable and for a time period sufficient to increase the numbers of said hematopoietic cell, said composition comprising a component characterized by:

(a) having a molecular weight in the range of 3 to 40 kD:

(b) being present in a crude preparation of native hCG or β-hCG; and

(c) having prohematopoietic activity *in vitro* as measured in an assay comprising:

(i) seeding isolated hematopoietic progenitor cells from normal bone marrow or cord blood cells in methylcellulose;

(ii) contacting said cells with said component; and

(iii) comparing the number of colonies formed after 10 days of culture from the cells contacted with the component with the number of colonies formed from cells not so contacted with the component,

wherein a higher number of colonies formed from said contacted cells indicates that the component has pro-hematopoietic activity;

said composition being active to increase proliferation of the cell.

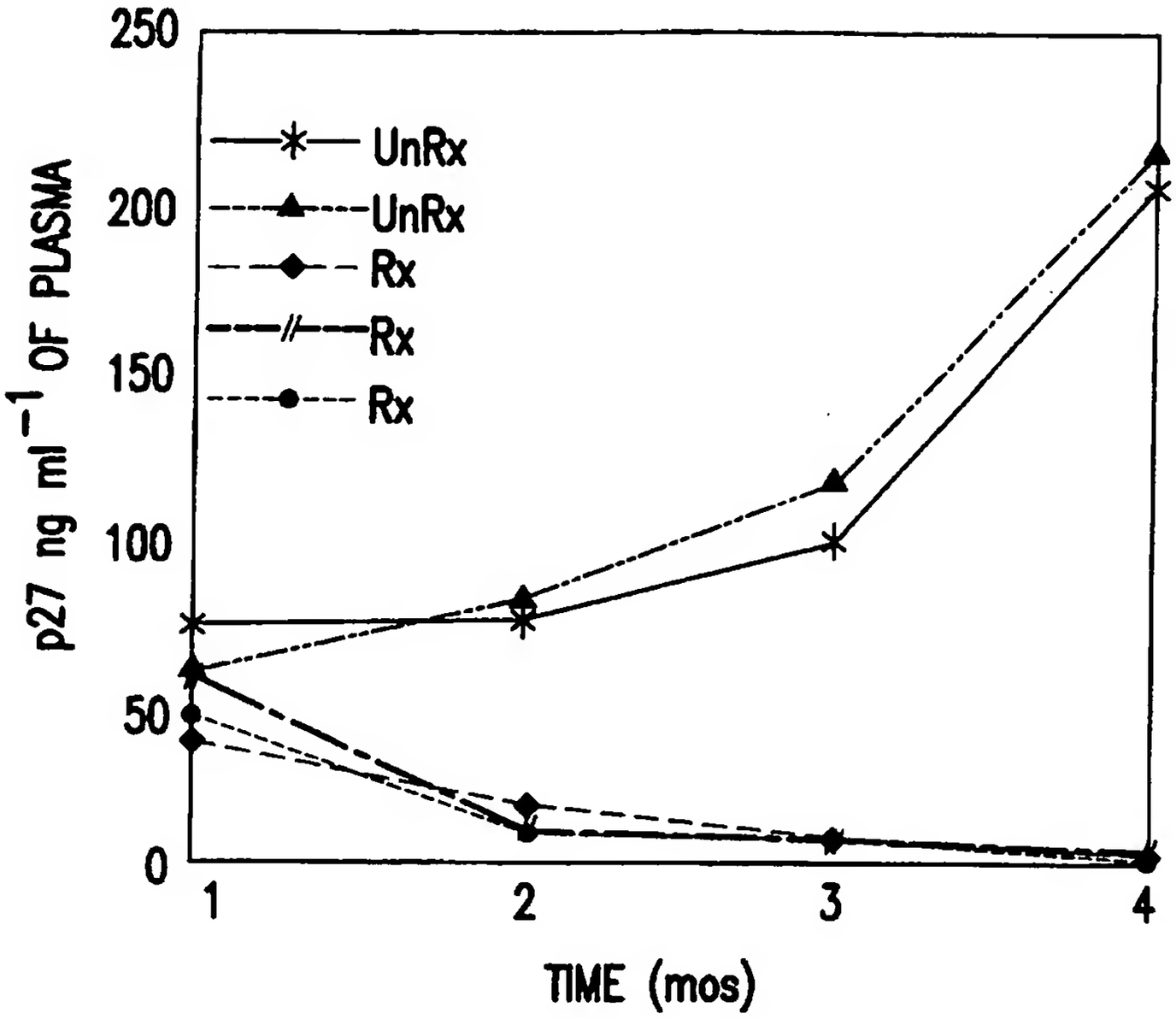
250
200
150
100
50
0
1
2
3
4
UnRx
UnRx
Rx
Rx
Rx
p27 ng ml^{-1} OF PLASMA
TIME (mos)

FIG.1A

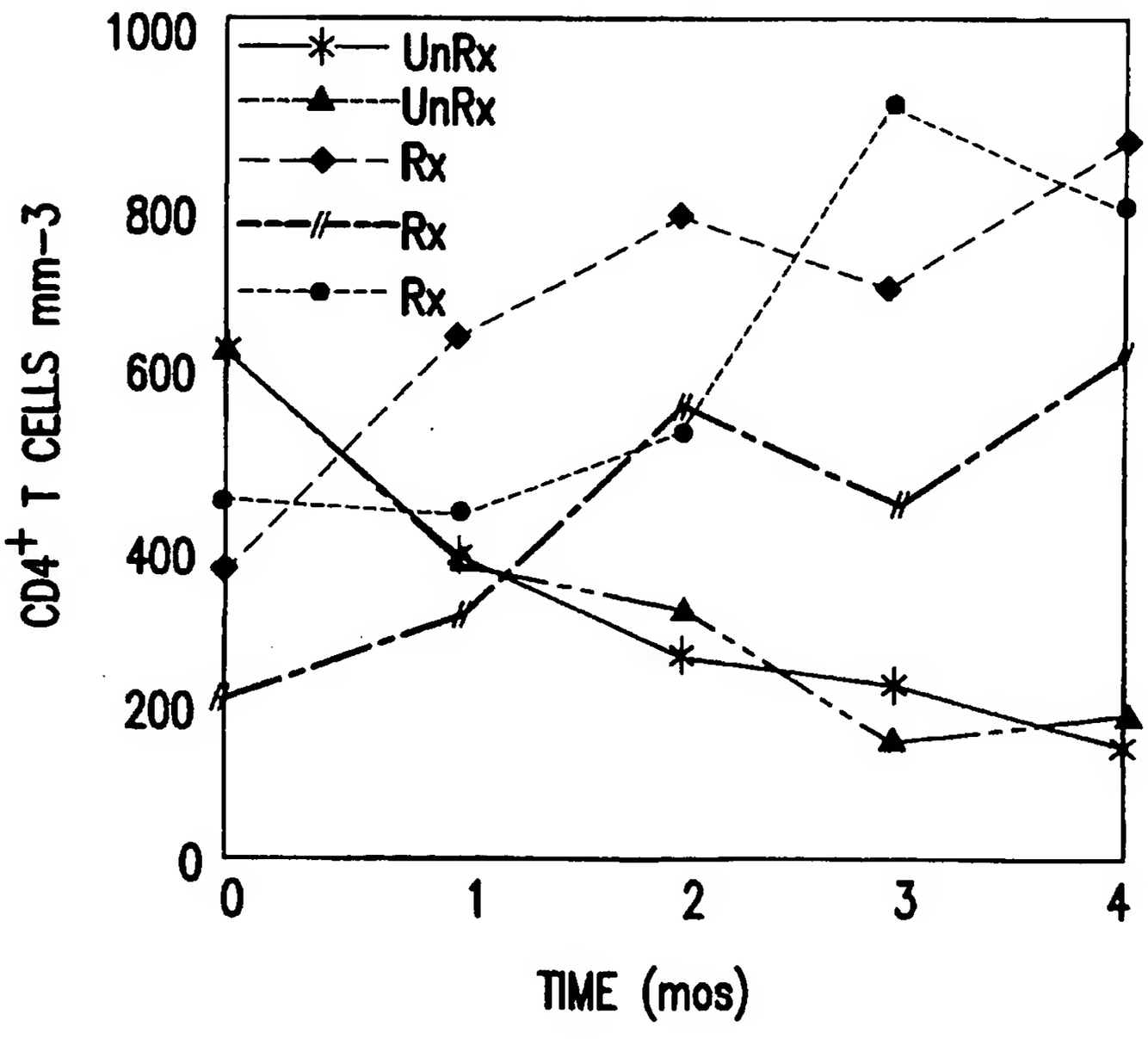
1000
800
600
400
200
0
0
1
2
3
4
UnRx
UnRx
Rx
Rx
Rx
CD4$^+$ T CELLS mm-3
TIME (mos)

FIG.1B

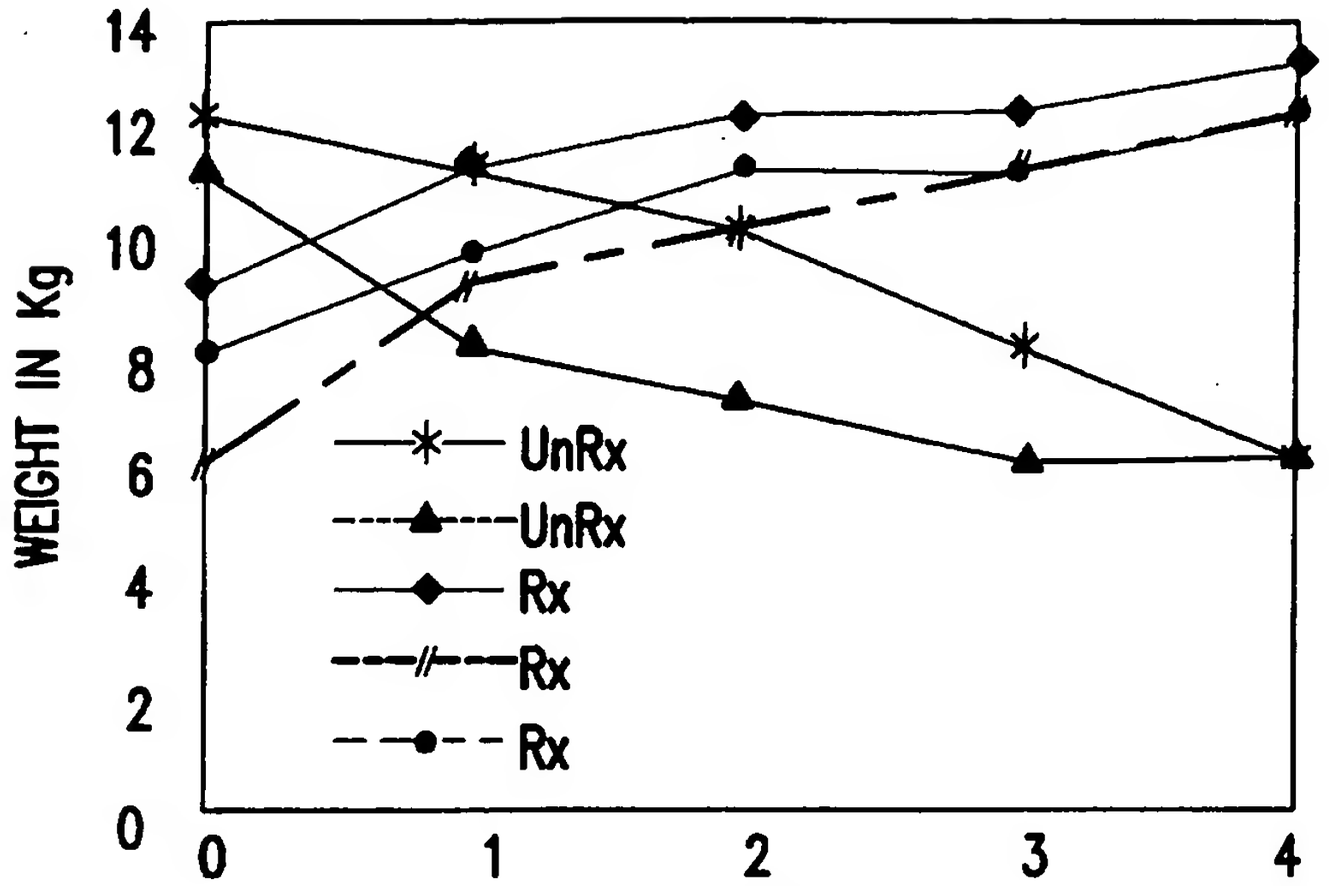
14
12
10
8
6
4
2
0
WEIGHT IN Kg
UnRx
UnRx
Rx
Rx
Rx
0
1
2
3
4
TIME (mos)

FIG.1C

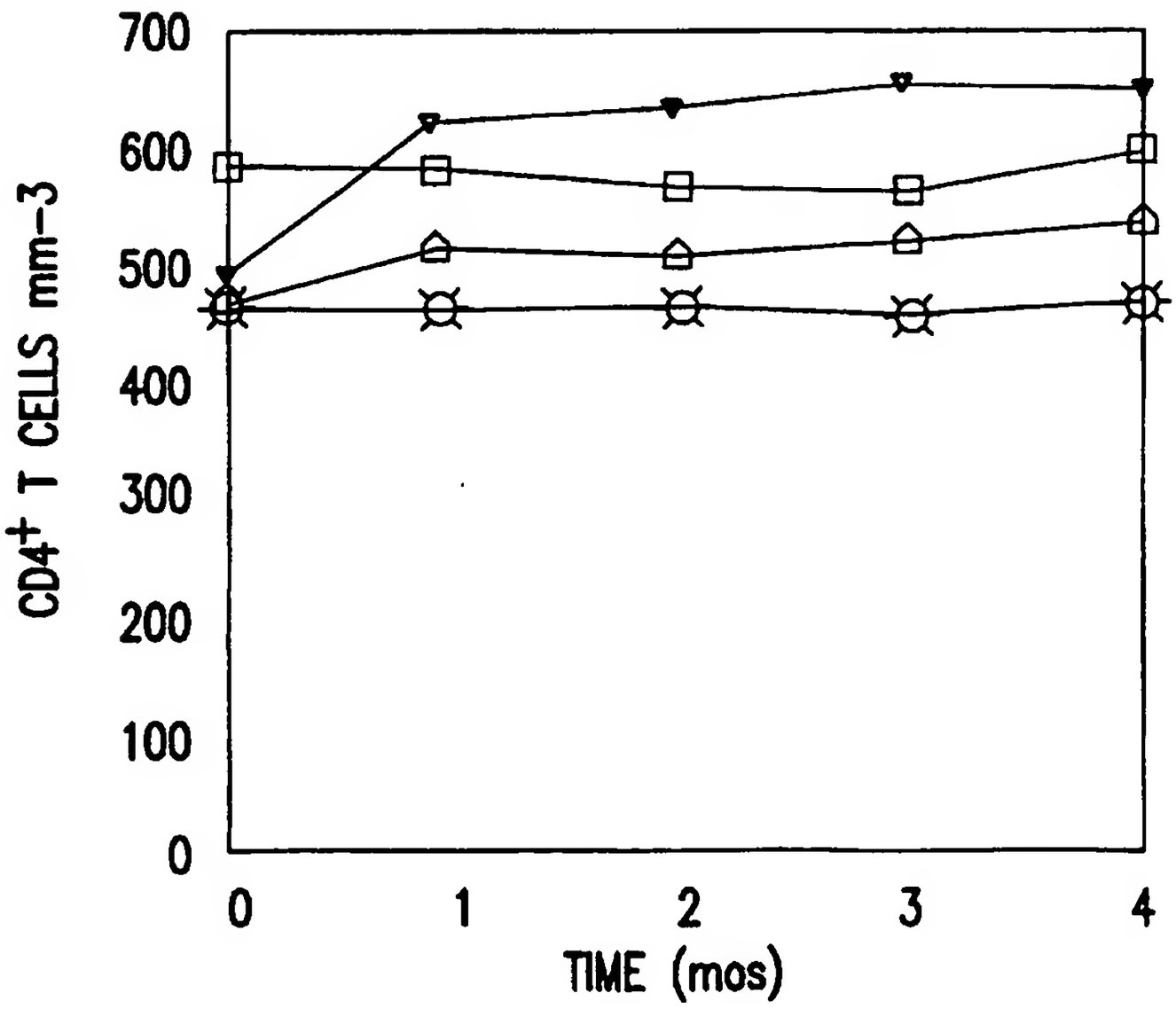
700
600
500
400
300
200
100
0
CD4+ T CELLS mm-3
0
1
2
3
4
TIME (mos)

FIG.1D

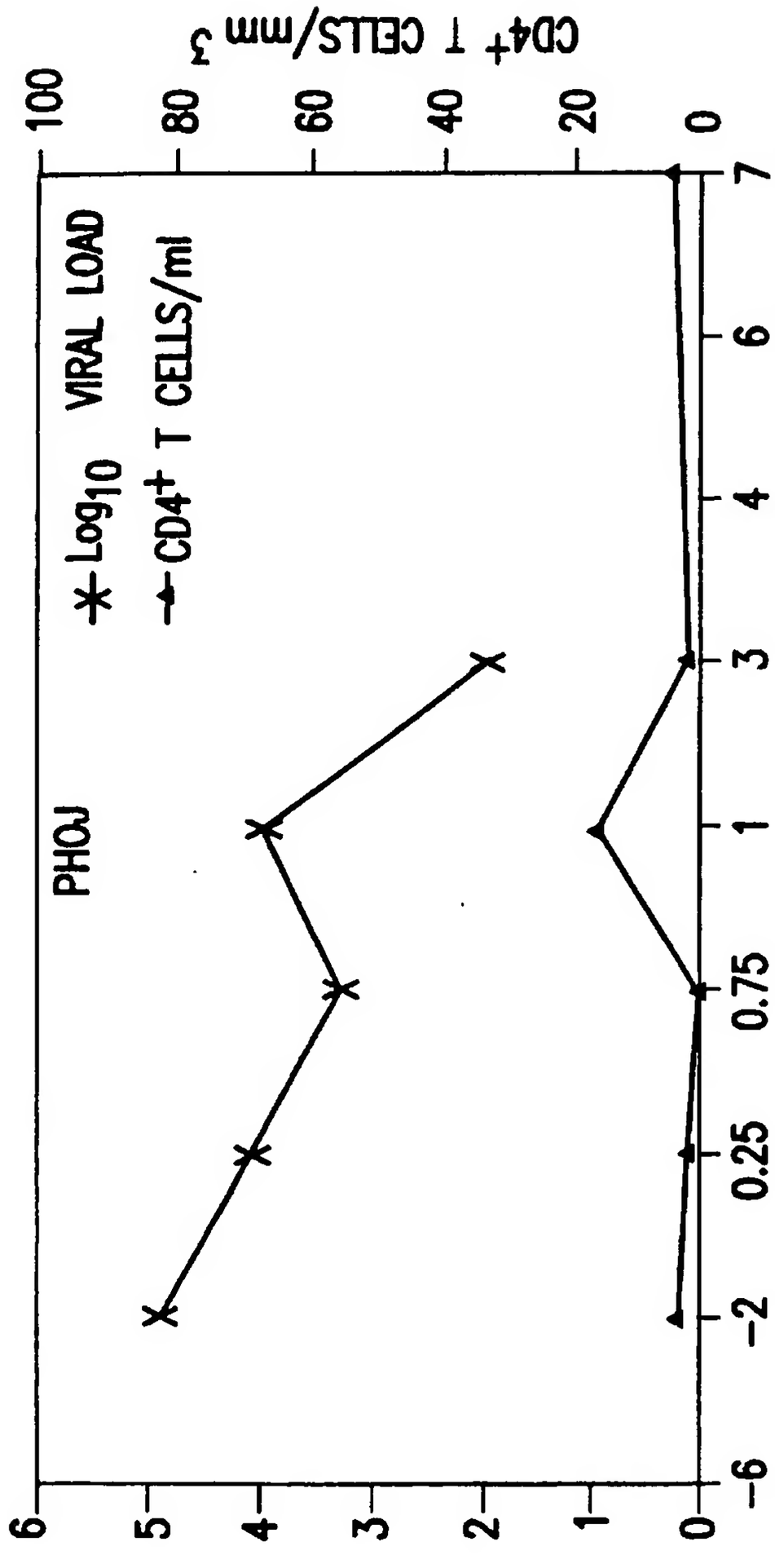
CD4+ T CELLS/mm 3
100
80
60
40
20
0
7
6
4
3
1
0.75
0.25
−2
−6
3tc,d4t,Norvir,
alpha−APA
TIONAL THERAPY
PHQJ
*Log10 VIRAL LOAD
+CD4+ T CELLS/ml
6
5
4
3
2
1
0
−6
Log10 VIRAL LOAD (RT−PCR)

FIG.2A

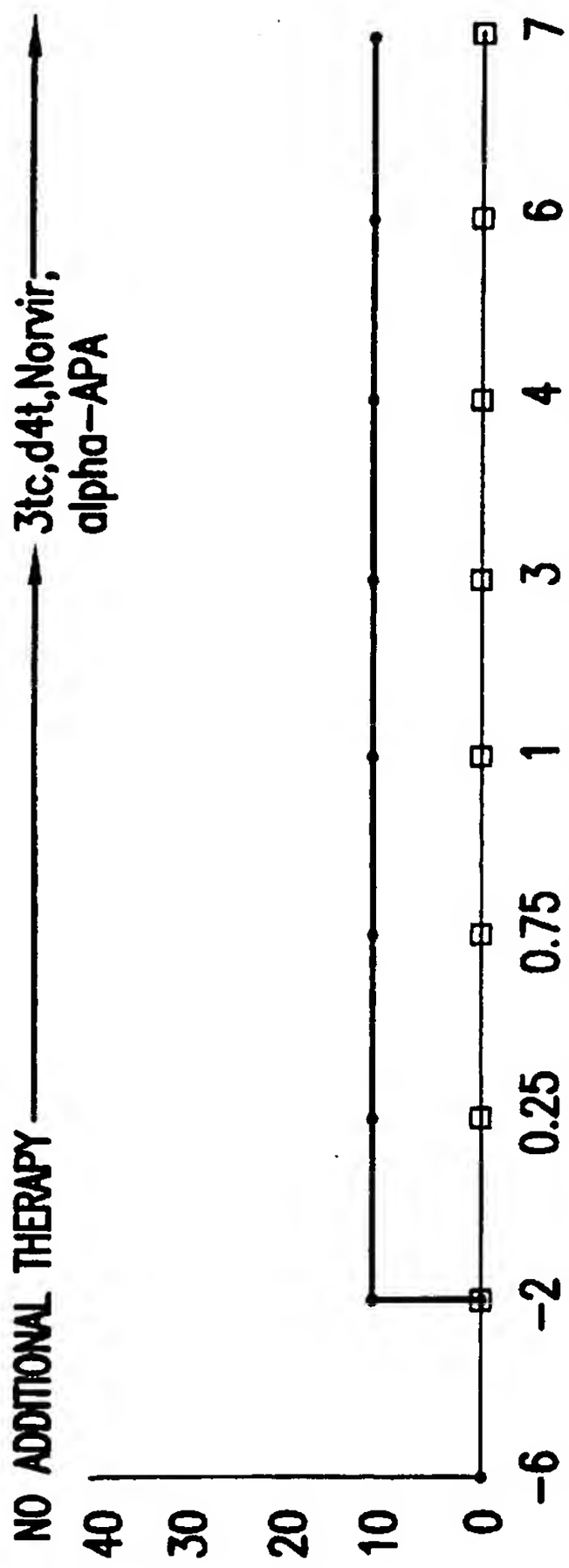
7
6
4
3
1
0.75
0.25
−2
−6
DURATION hCG TREATMENT (MONTHS)
NO ADDITIONAL THERAPY
40
30
20
10
0
IU hCG/WEEK*1,000

FIG.2B

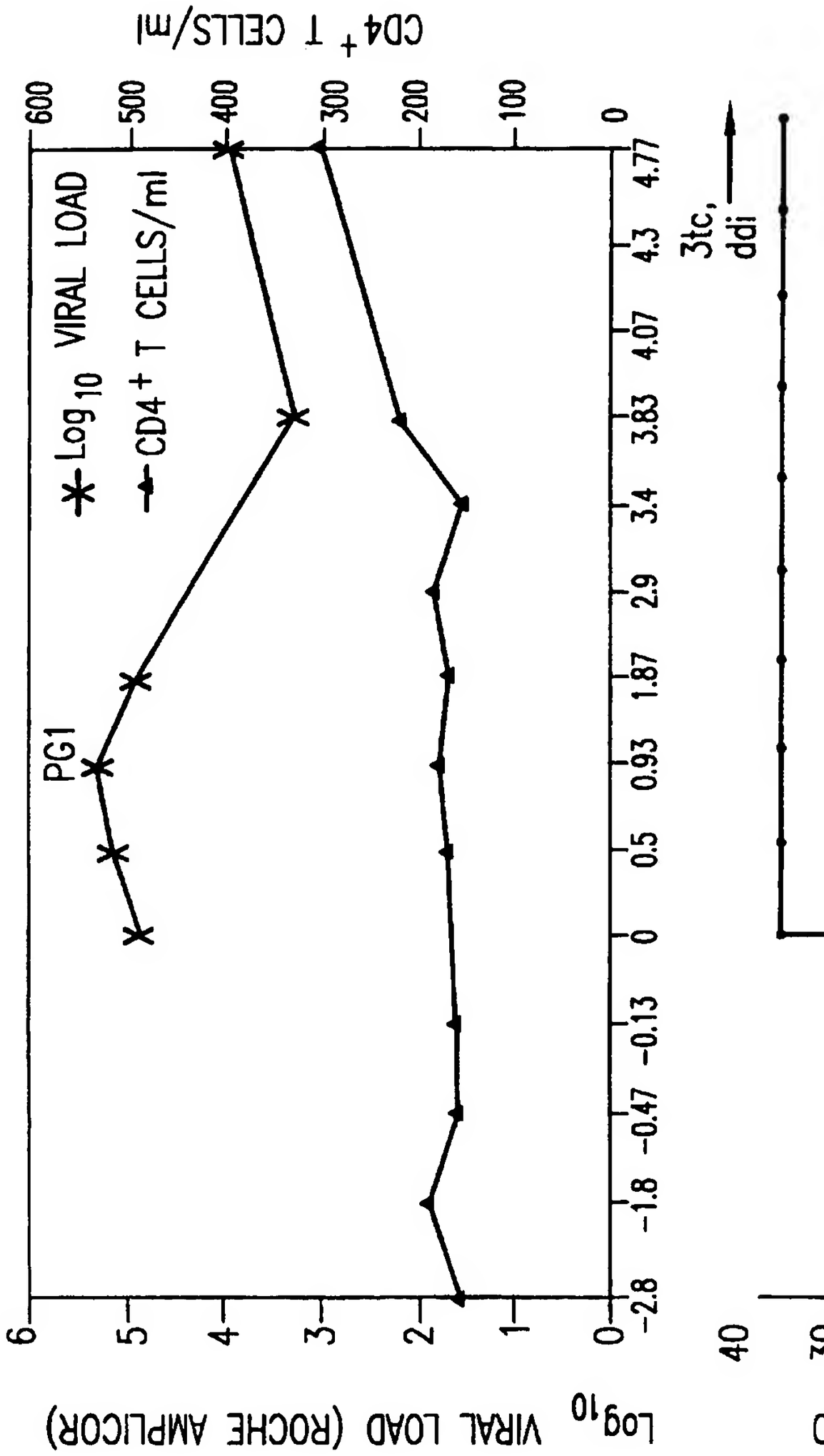
CD4+ T CELLS/ml
600
500
400
300
200
100
0
Log10 VIRAL LOAD
CD4+ T CELLS/ml
PG1
3tc, ddi
4.77
4.3
4.07
3.83
3.4
2.9
1.87
0.93
0.5
0
−0.13
−0.47
−1.8
−2.8
6
5
4
3
2
1
0
Log10 VIRAL LOAD (ROCHE AMPLICOR)

FIG.2C

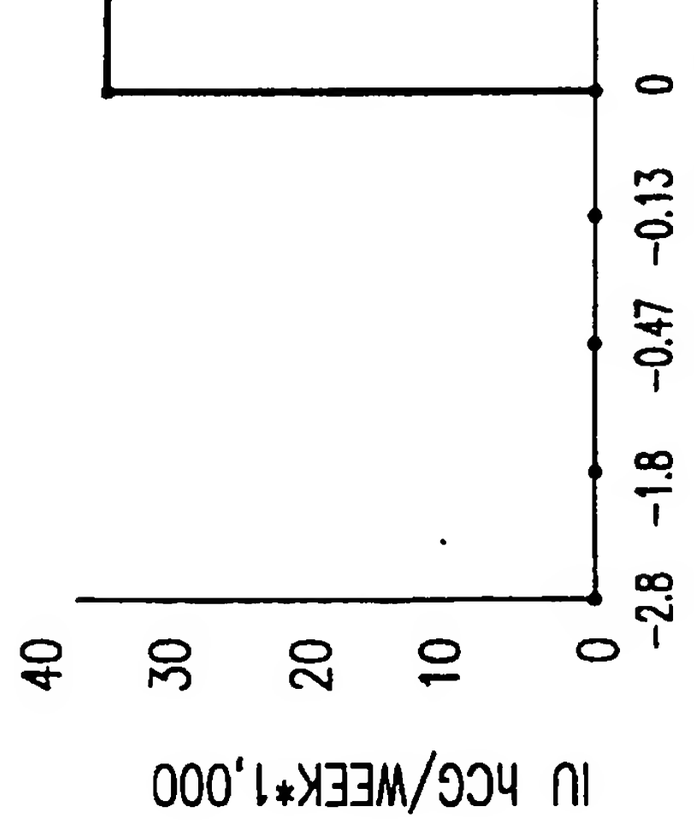
4.77
4.3
4.07
3.83
3.4
2.9
1.87
0.93
0.5
0
−0.13
−0.47
−1.8
−2.8
DURATION OF hCG TREATMENT (MONTHS)
40
30
20
10
0
IU hCG/WEEK*1,000

FIG.2D

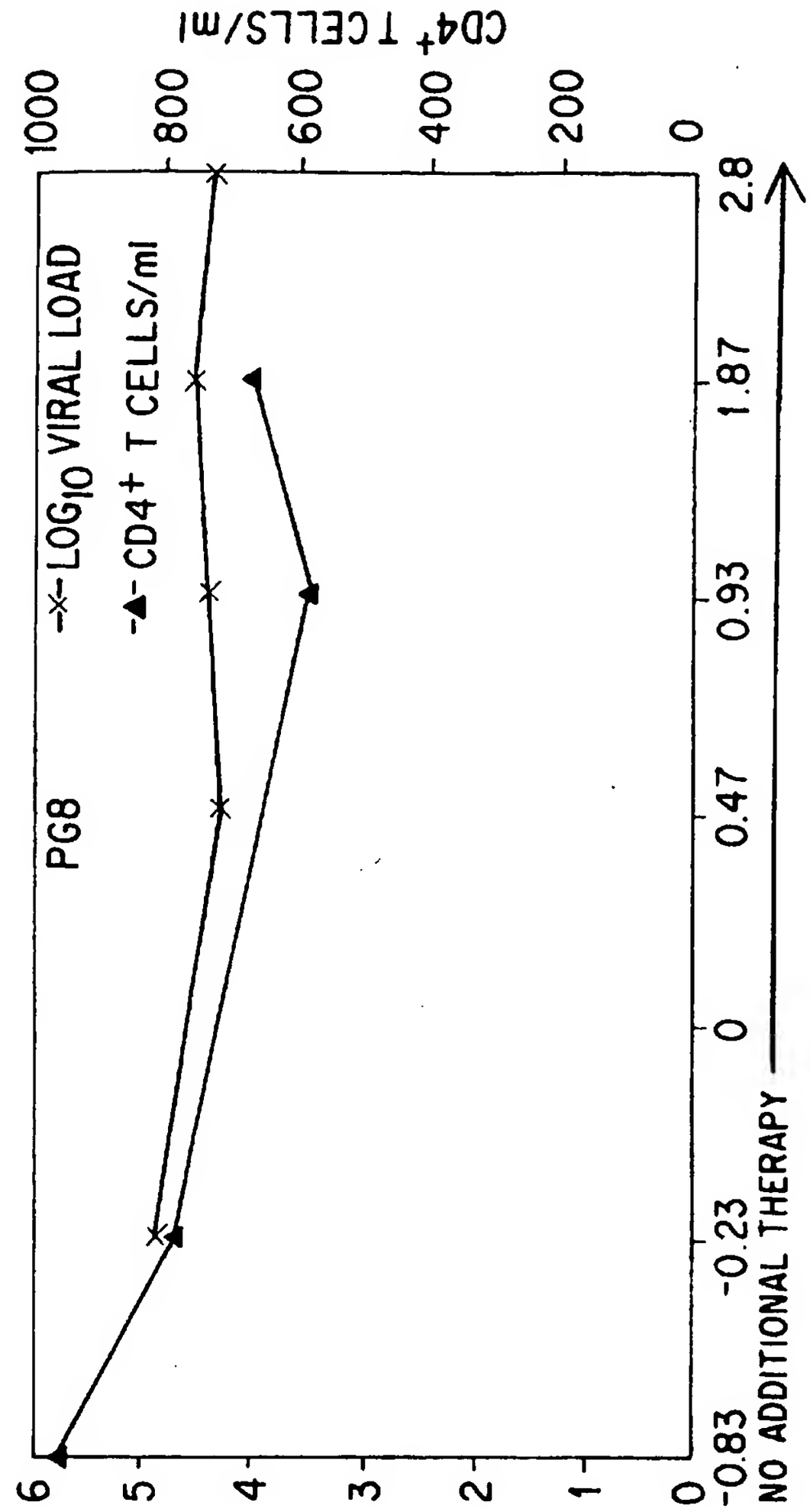
CD4+ T CELLS/ml
1000
800
600
400
200
0
PG8
-×- LOG10 VIRAL LOAD
-▲- CD4+ T CELLS/ml
6
5
4
3
2
1
0
2.8
1.87
0.93
0.47
0
-0.23
-0.83
NO ADDITIONAL THERAPY
LOG10 VIRAL LOAD (ROCHE AMPLICOR)

FIG.2E

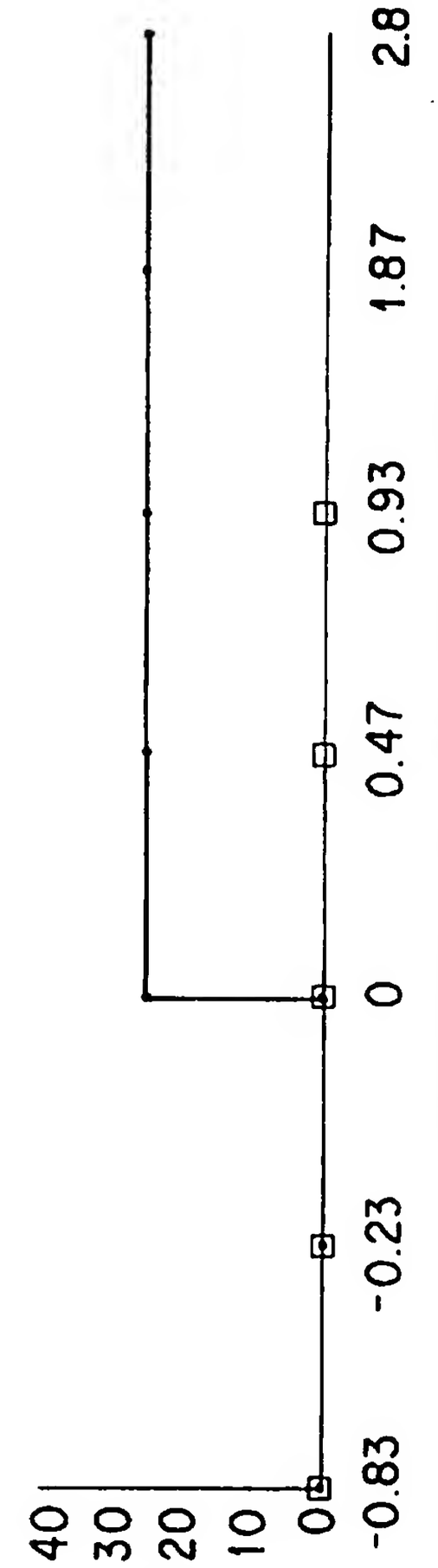
2.8
1.87
0.93
0.47
0
-0.23
-0.83
DURATION OF hCG TREATMENT (MOS)
40
30
20
10
0
IU hCG/WEEK*1,000

FIG.2F

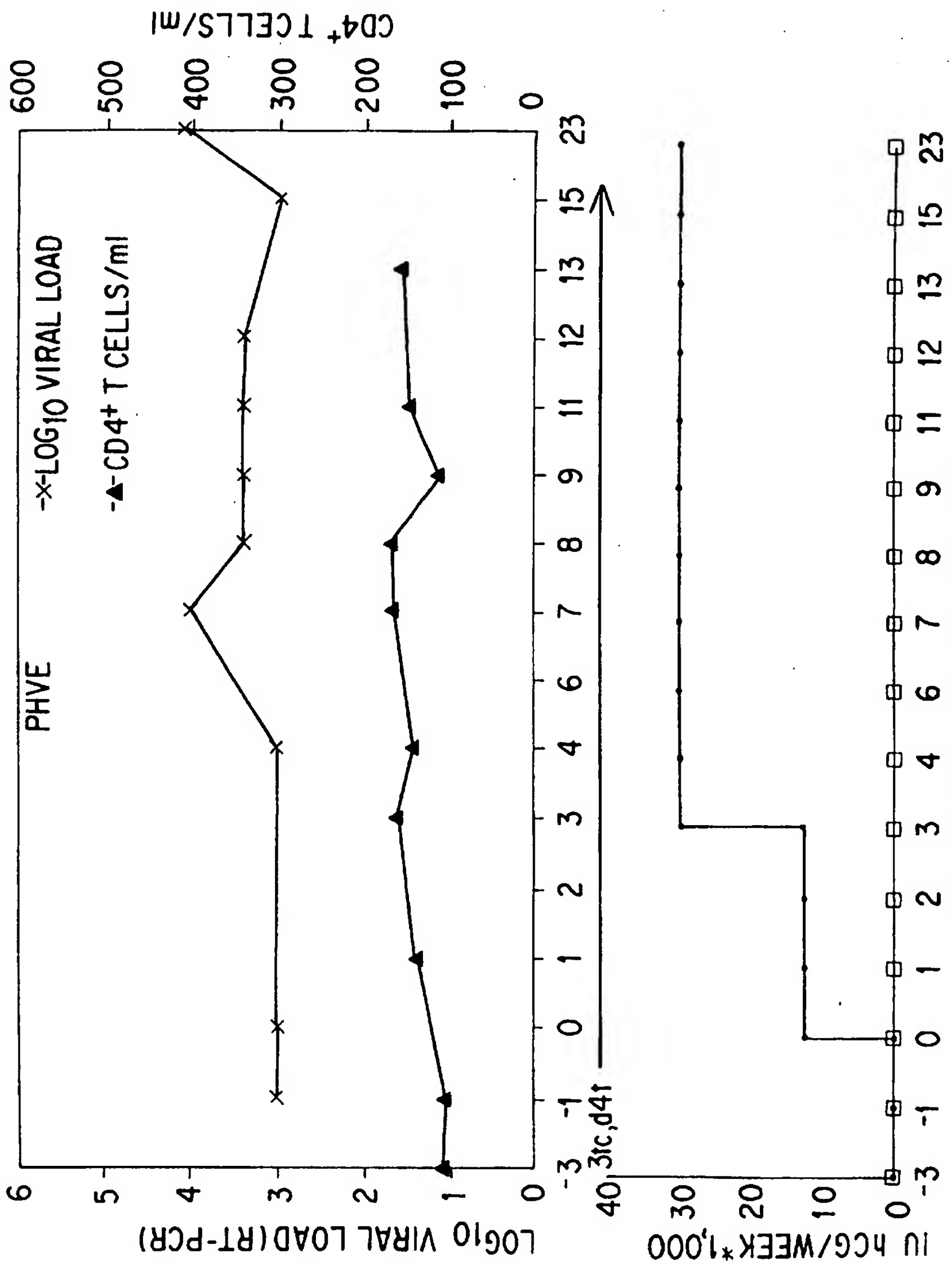
CD4+ T CELLS/ml
600
500
400
300
200
100
0
23
15
13
12
11
9
8
7
6
4
3
2
1
0
-1
-3
PHVE
-X-LOG10 VIRAL LOAD
-▲-CD4+ T CELLS/ml
6
5
4
3
2
1
0
LOG10 VIRAL LOAD (RT-PCR)
3tc,d4t
40
30
20
10
0
IU hCG/WEEK*1,000
DURATION OF hCG TREATMENT (MOS)

FIG.2G

FIG.2H

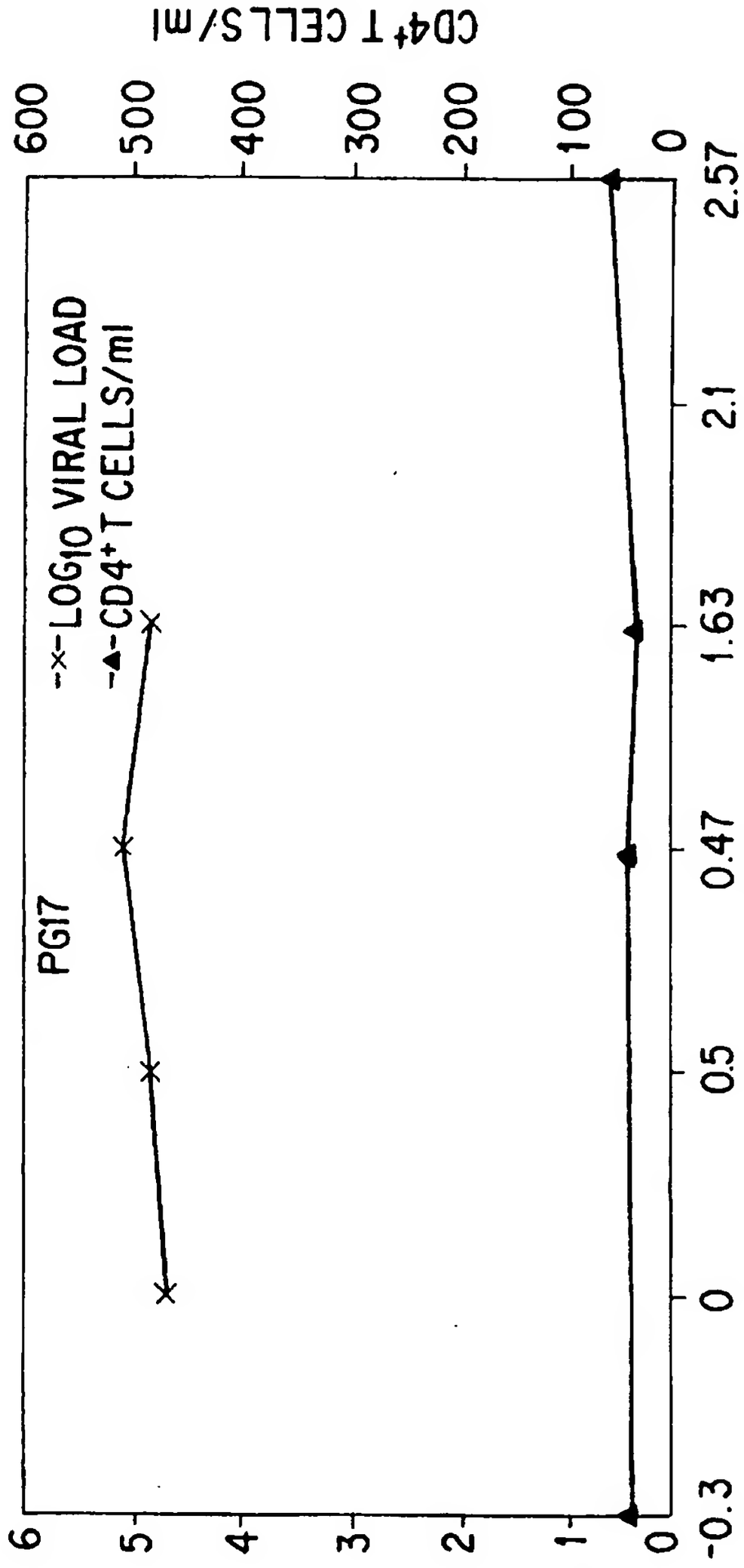
CD4+ T CELLS/ml
600
500
400
300
200
100
0
2.57
2.1
1.63
0.47
0
-0.3
-x- LOG10 VIRAL LOAD
-▲- CD4+ T CELLS/ml
PG17
NO ADDITIONAL THERAPY
LOG10 VIRAL LOAD (ROCHE AMPLICOR)
6
5
4
3
2
1
0

FIG.2I

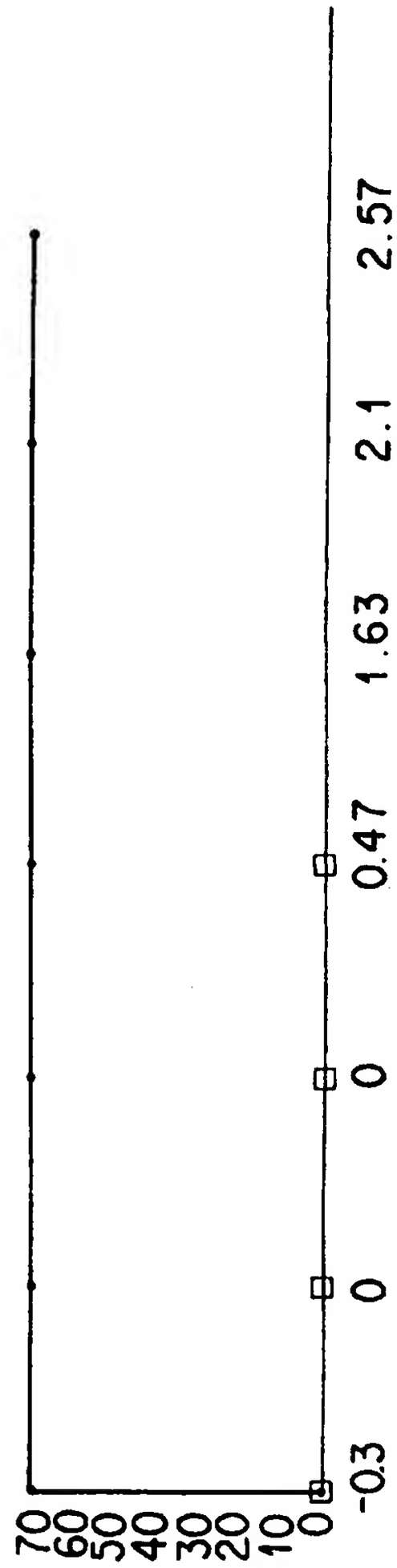
2.57
2.1
1.63
0.47
0
-0.3
DURATION OF hCG TREATMENT(MOS)
IU hCG/WEEK*1,000
70
60
50
40
30
20
10
0

FIG.2J

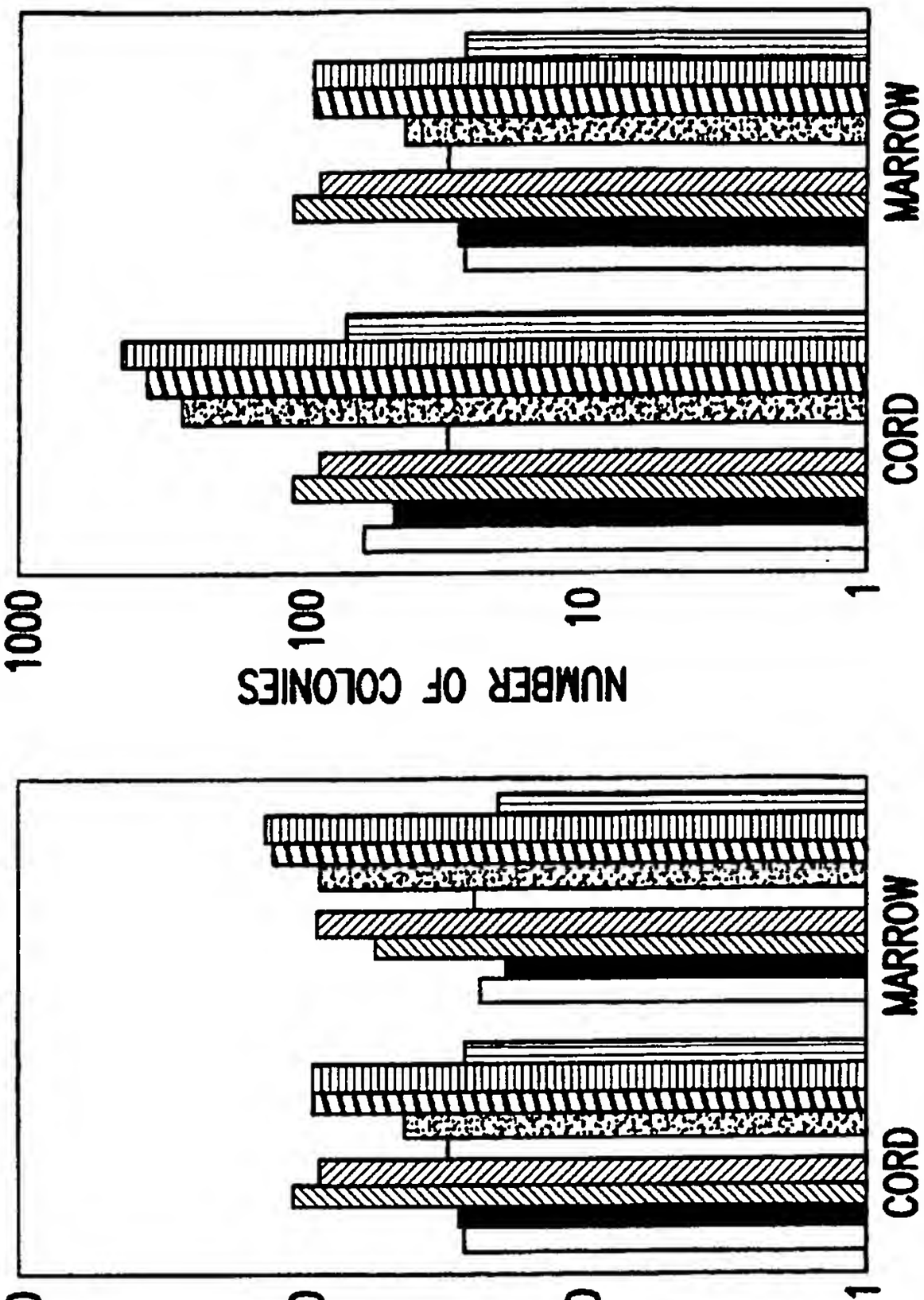

FIG.3C

FIG.3B

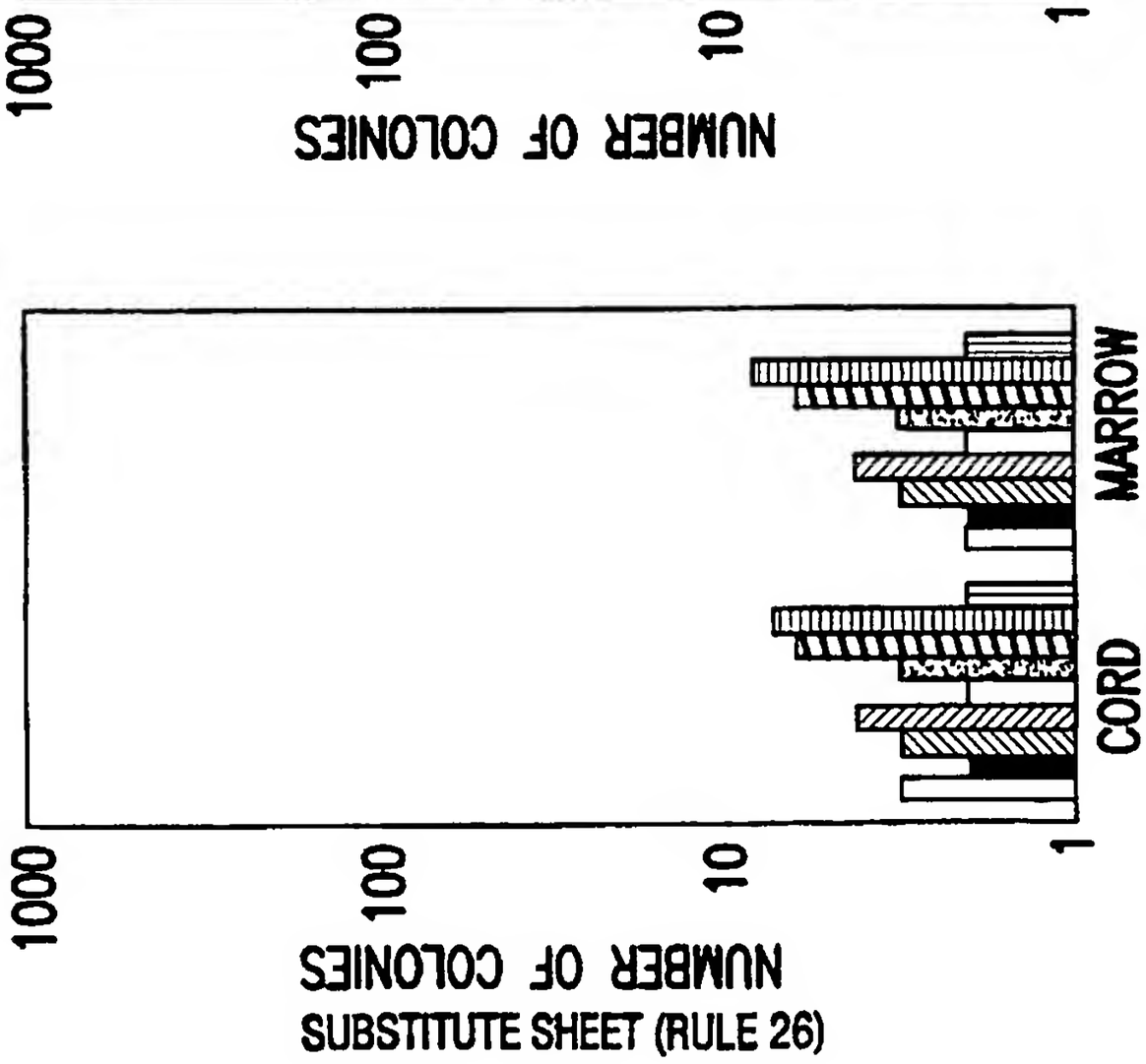

FIG.3A

```
AGACAAGGCA GGGGACGCAC CAAGG ATG GAG ATG TTC CAG GGG CTG CTG CTG     52
                            Met Glu Met Phe Gln Gly Leu Leu Leu
                            -20                 -15
TTG CTG CTG CTG AGC ATG GGC GGG ACA TGG GCA TCC AAG GAG CCG CTT    100
Leu Leu Leu Leu Ser Met Gly Gly Thr Trp Ala Ser Lys Glu Pro Leu
    -10                 -5                  1                   5
CGG CCA CGG TGC CGC CCC ATC AAT GCC ACC CTG GCT GTG GAG AAG GAG    148
Arg Pro Arg Cys Arg Pro Ile Asn Ala Thr Leu Ala Val Glu Lys Glu
                    10                  15                  20
GGC TGC CCC GTG TGC ATC ACC GTC AAC ACC ACC ATC TGT GCC GGC TAC    196
Gly Cys Pro Val Cys Ile Thr Val Asn Thr Thr Ile Cys Ala GLy Tyr
                25                  30                  35
TGC CCC ACC ATG ACC CGC GTG CTG CAG GGG GTC CTG CCG GCC CTG CCT    244
Cys Pro Thr Met Thr Arg Val Leu Gln Gly Val Leu Pro Ala Leu Pro
            40                  45                  50
CAG CTG GTG TGC AAC TAC CGC GAT GTG CGC TTC GAG TCC ATC CGG CTC    292
Gln Val Val Cys Asn Tyr Arg Asp Val Arg Phe Glu Ser Ile Arg Leu
        55                  60                  65
CCT GGC TGC CCG CGC GGC GTG AAC CCC GTG GTC TCC TAC GCC GTG GCT    340
Pro Gly Cys Pro Arg Gly Val Asn Pro Val Val Ser Tyr Ala Val Ala
    70                  75                  80                  85
CTC AGC TGT CAA TGT GCA CTC TGC CGC CGC AGC ACC ACT GAC TGC GGG    388
Leu Ser Cys Gln Cys Ala Leu Cys Arg Arg Ser THr Thr Asp Cys Gly
                    90                  95                  100
GGT CCC AAG GAC CAC CCC TTG ACC TGT GAT GAC CCC CGC TTC CAG GAC    436
Gly Pro Lys Asp His Pro Leu Thr Cys Asp Asp Pro Arg Phe Gln Asp
                105                 110                 115
TCC TCT TCC TCA AAG GCC CCT CCC CCC AGC CTT CCA AGC CCA TCC CGA    484
Ser Ser Ser Ser Lys Ala Pro Pro Pro Ser Leu Pro Ser Pro Ser Arg
            120                 125                 130
CTC CCG GGG CCC TCG GAC ACC CCG ATC CTC CCA CAA TAAAGGCTTC         530
Leu Pro Gly Pro Ser Asp Thr Pro Ile Leu Pro Gln
        135                 140                 145
TCAATCCGC                                                          539
```

FIG.4

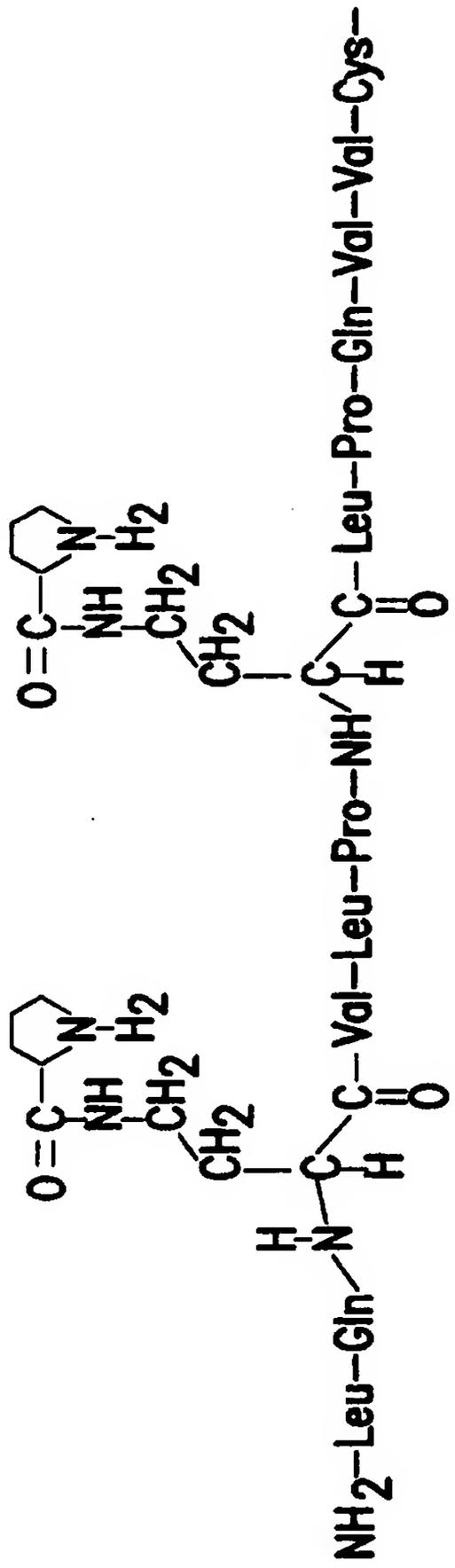
NH2–Leu–Gln–
–Val–Leu–Pro–NH–
–Leu–Pro–Gln–Val–Val–Cys–

FIG.5A

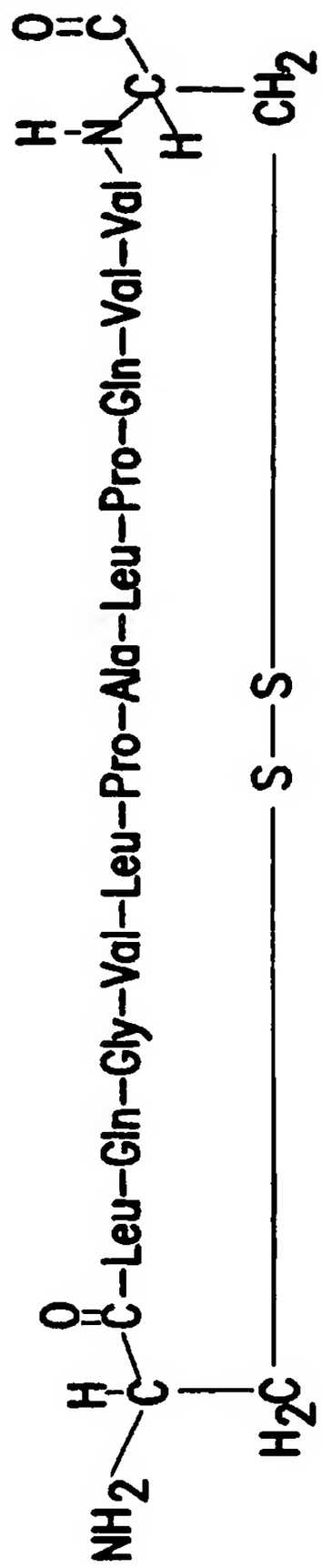
–Gln–Gly–Val–Leu–Pro–Ala–Leu–Pro–Gln–Val–Val–
S—S
NH2

FIG.5B

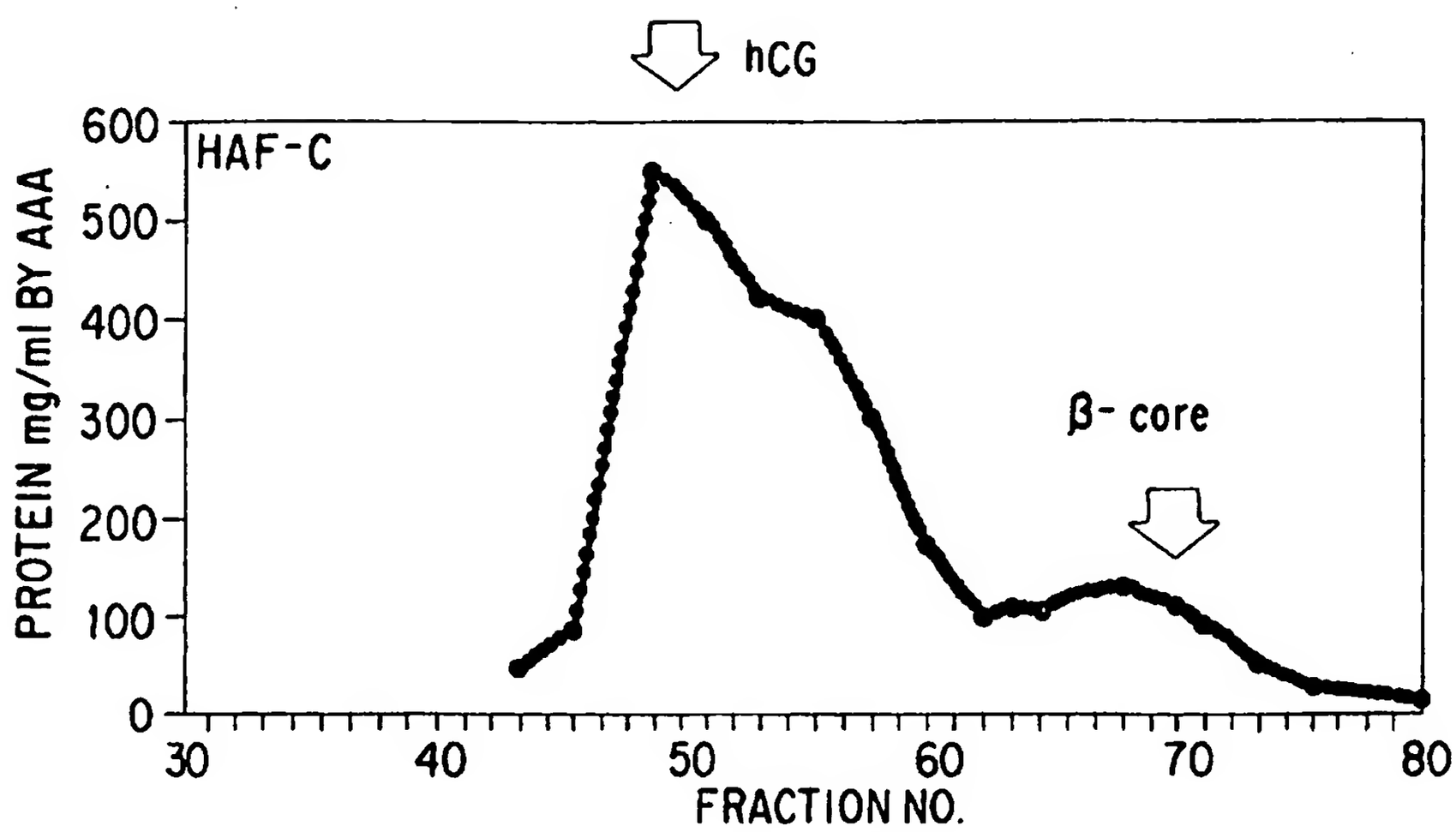
hCG
HAF-C
β- core
PROTEIN mg/ml BY AAA
600
500
400
300
200
100
0
30
40
50
60
70
80
FRACTION NO.

FIG. 6A

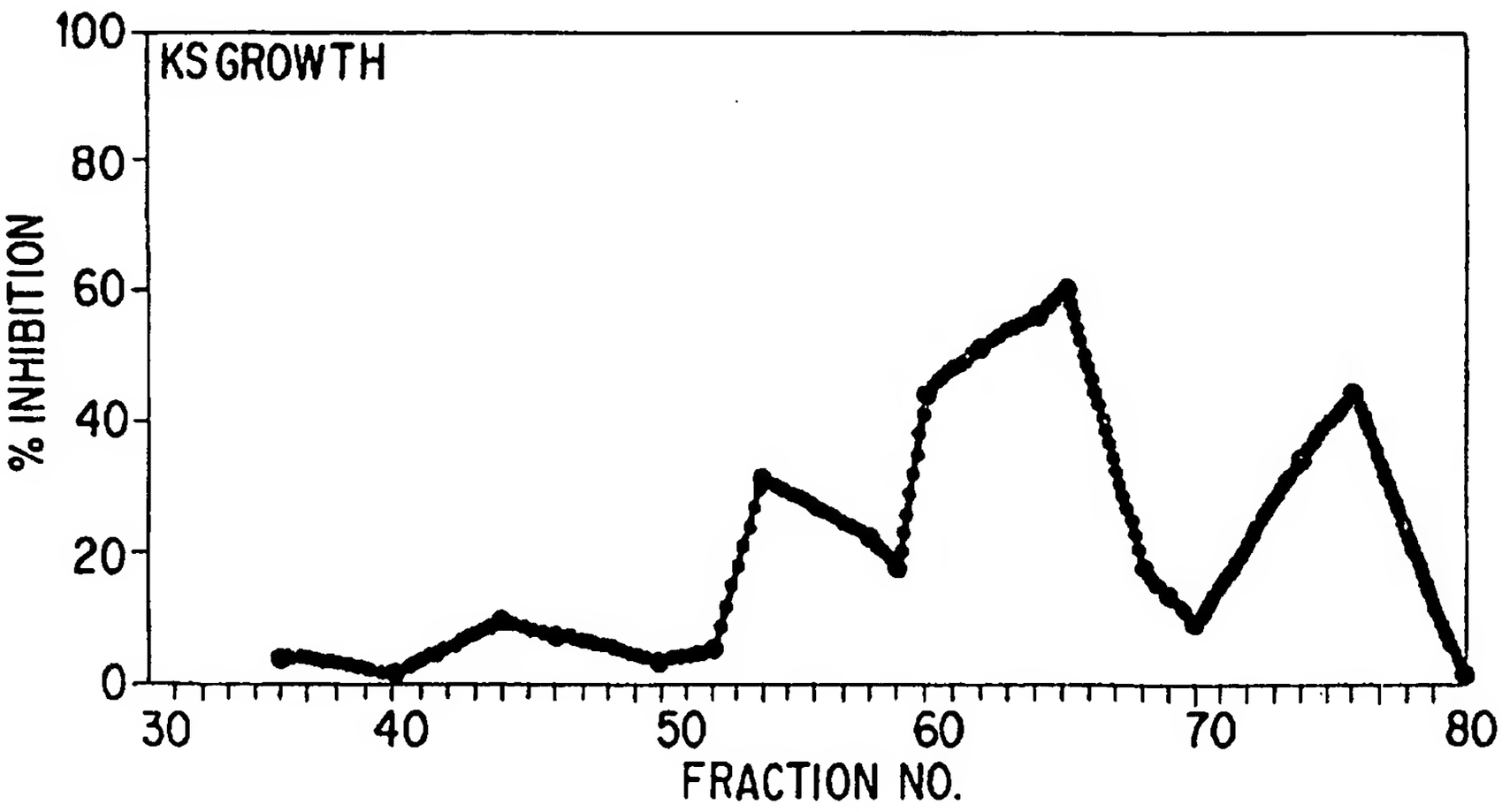
KS GROWTH
% INHIBITION
100
80
60
40
20
0
30
40
50
60
70
80
FRACTION NO.

FIG. 6B

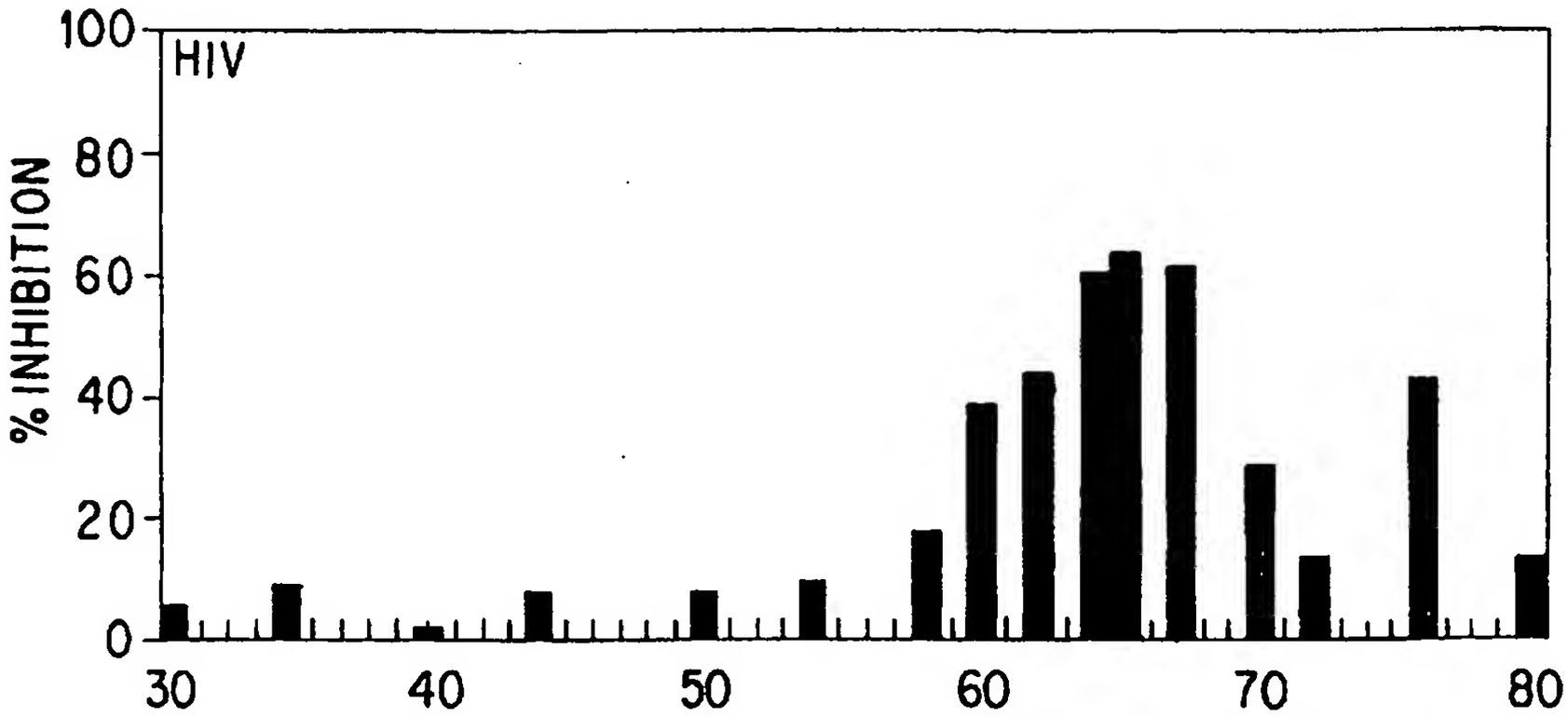
HIV
% INHIBITION
100
80
60
40
20
0
30
40
50
60
70
80
FRACTION NO.

FIG.6C

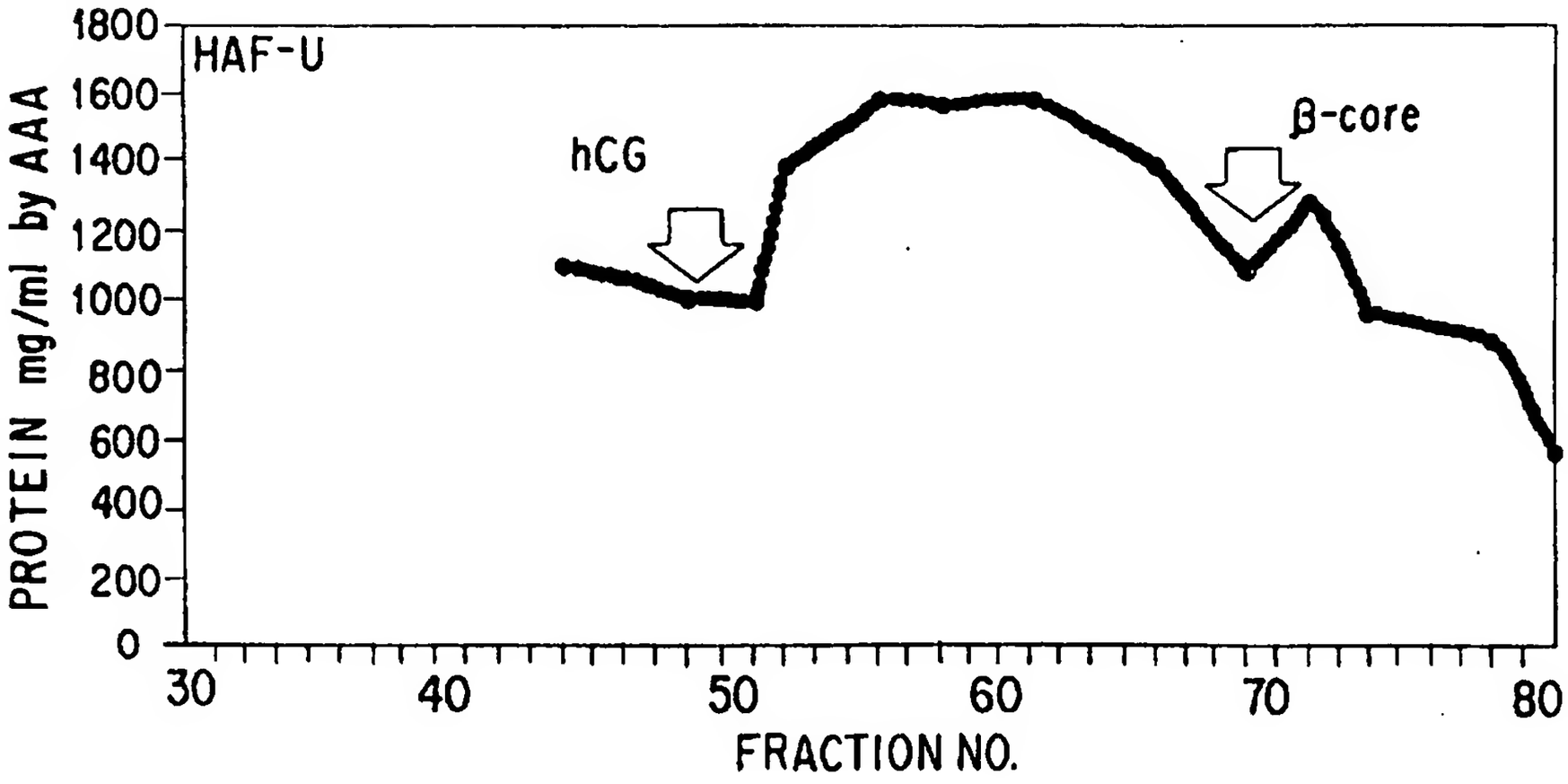
HAF-U
PROTEIN mg/ml by AAA
1800
1600
1400
1200
1000
800
600
400
200
0
hCG
β-core
30
40
50
60
70
80
FRACTION NO.

FIG.6D

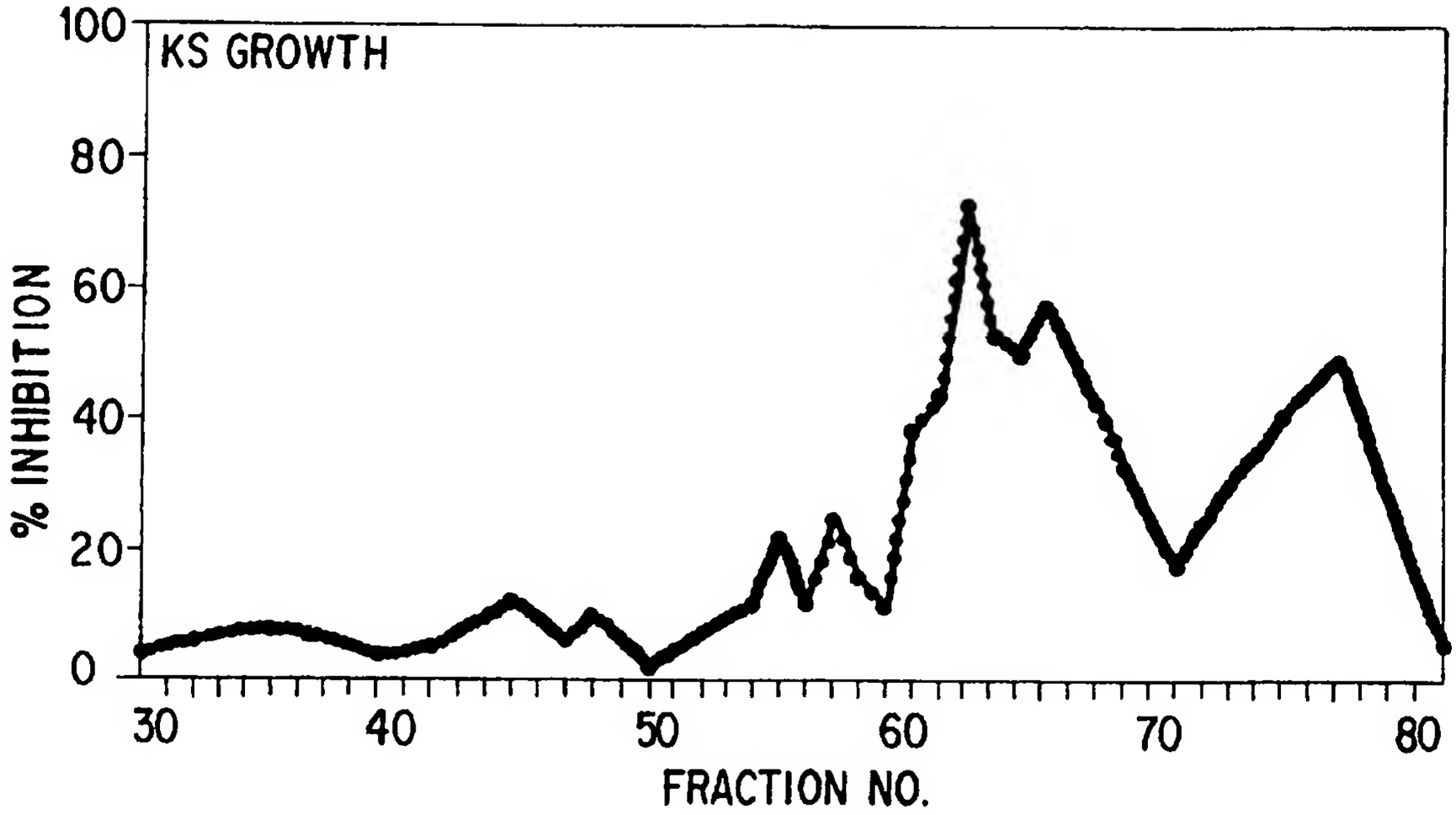
KS GROWTH
% INHIBITION
100
80
60
40
20
0
30
40
50
60
70
80
FRACTION NO.

FIG.6E

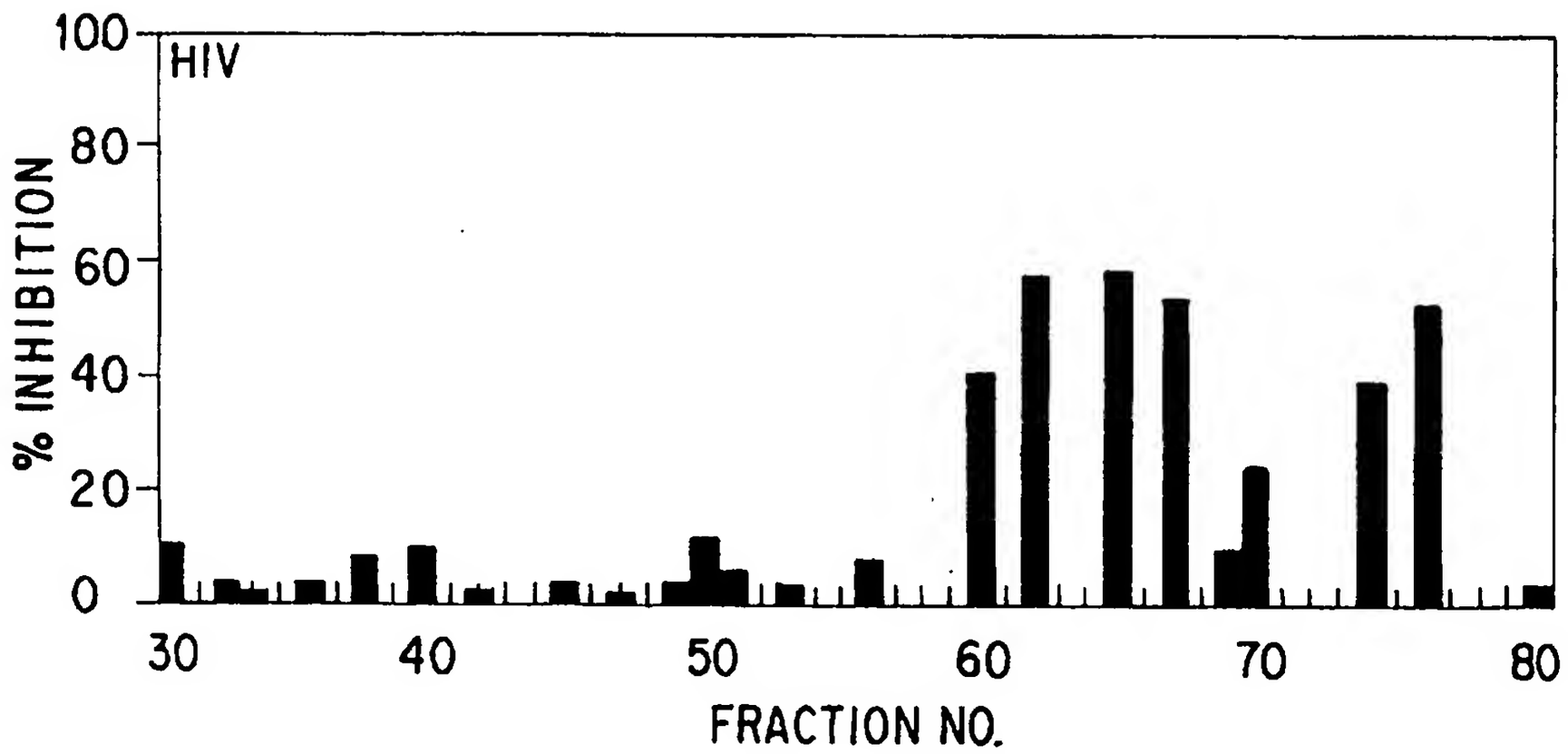
HIV
% INHIBITION
100
80
60
40
20
0
30
40
50
60
70
80
FRACTION NO.

FIG.6F

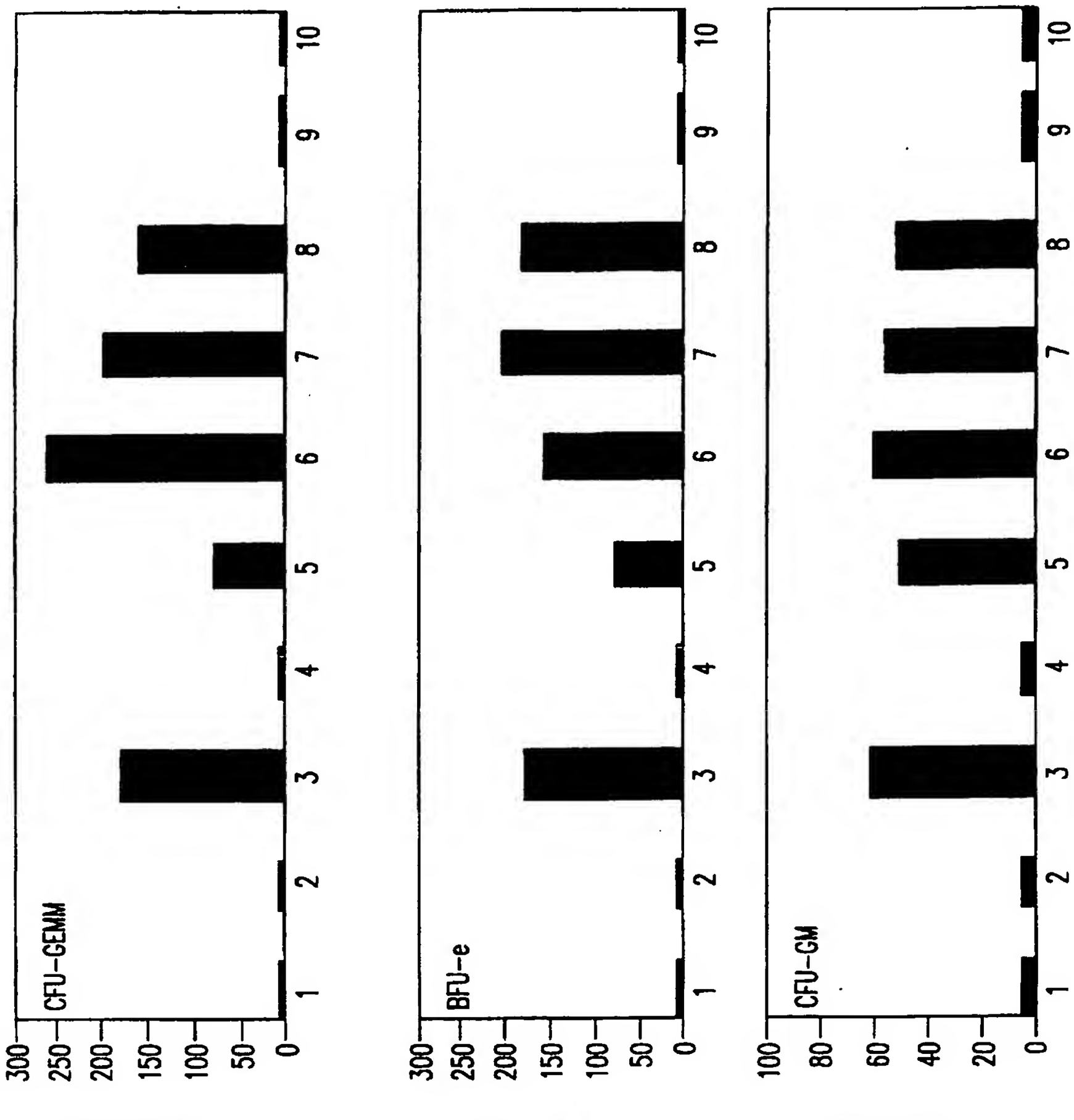

FIG.7A FIG.7B FIG.7C

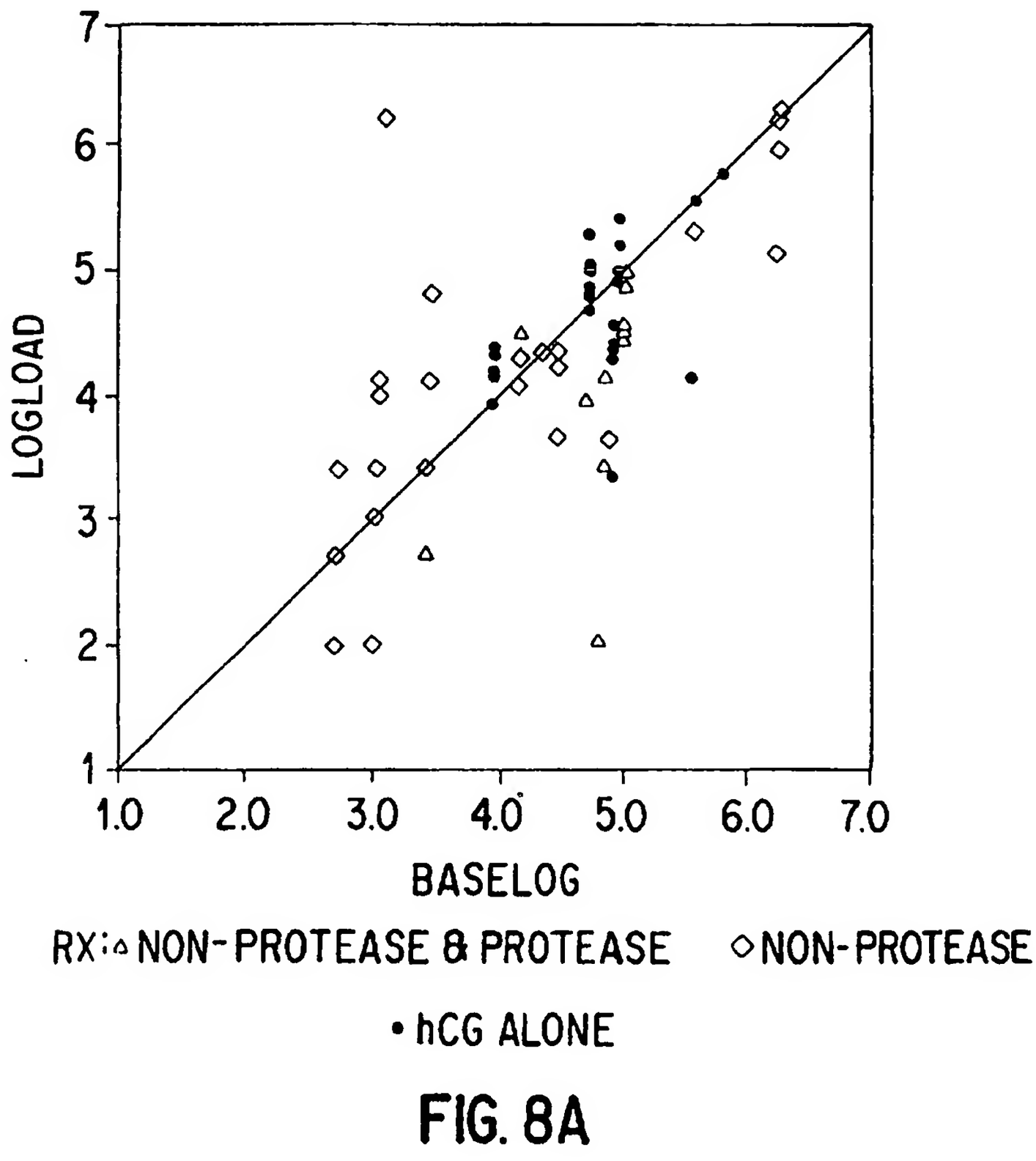
LOGLOAD
7
6
5
4
3
2
1
1.0
2.0
3.0
4.0
5.0
6.0
7.0
BASELOG
RX: △ NON-PROTEASE & PROTEASE
◇ NON-PROTEASE
• hCG ALONE

FIG. 8A

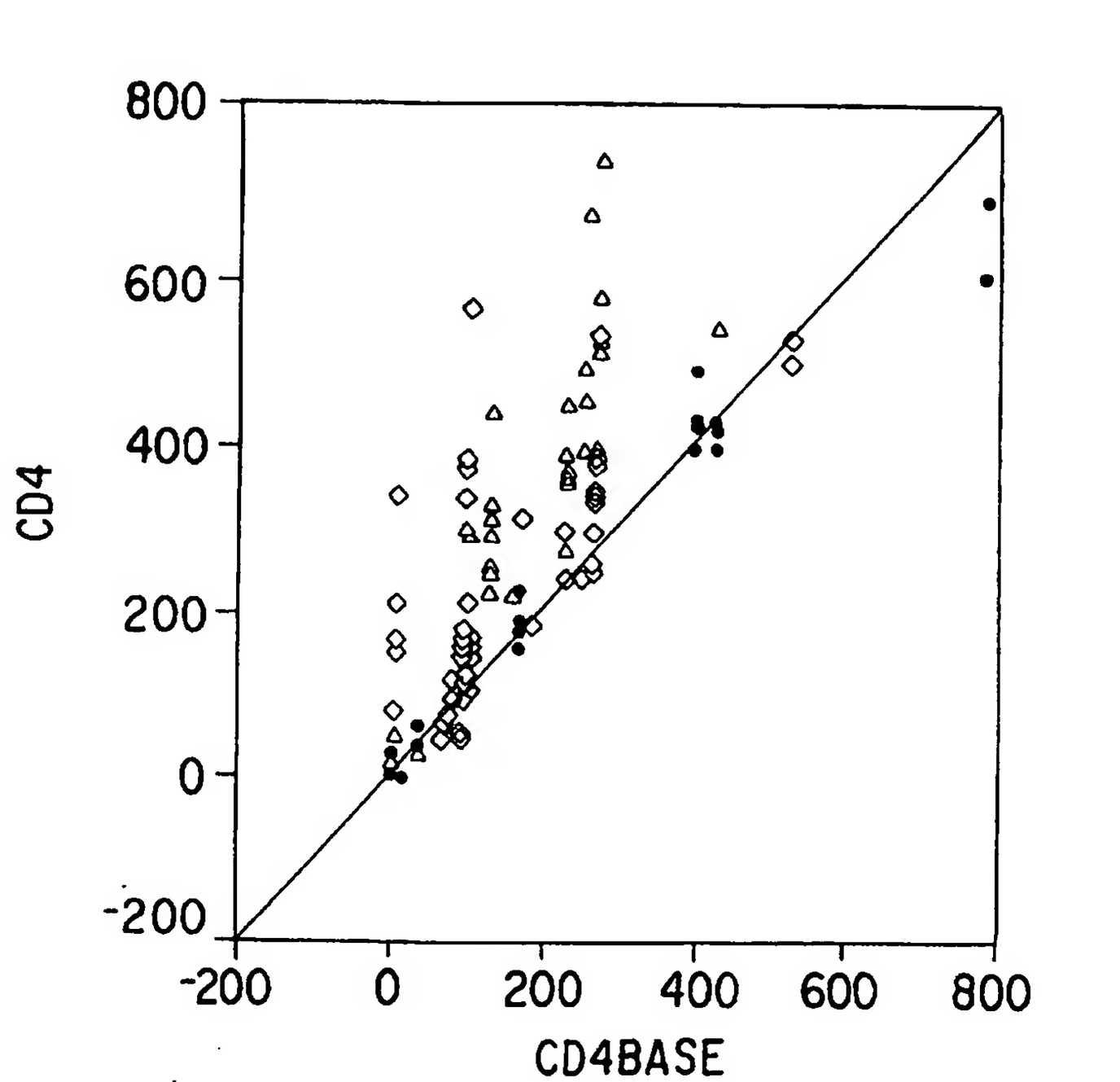
800
600
400
200
0
-200
CD4
-200
0
200
400
600
800
CD4BASE
RX: △ NON-PROTEASE & PROTEASE
△ NON-PROTEASE
• hCG ALONE

FIG. 8B

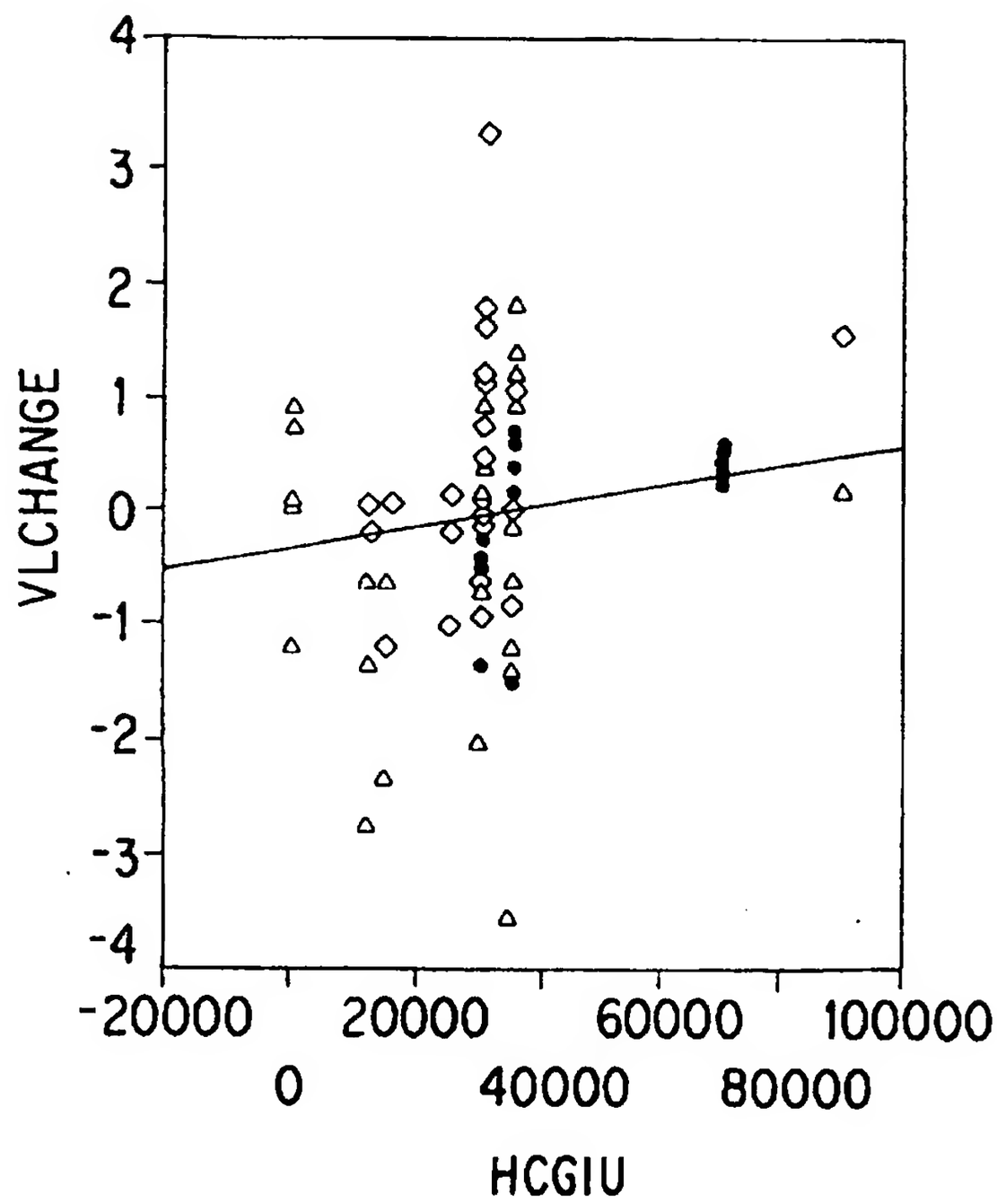
VLCHANGE
4
3
2
1
0
-1
-2
-3
-4
-20000
0
20000
40000
60000
80000
100000
HCGIU
RX: △ NON-PROTEASE & PROTEASE ◇ NON-PROTEASE
• hCG ALONE —TOTAL POPULATION

FIG. 8C

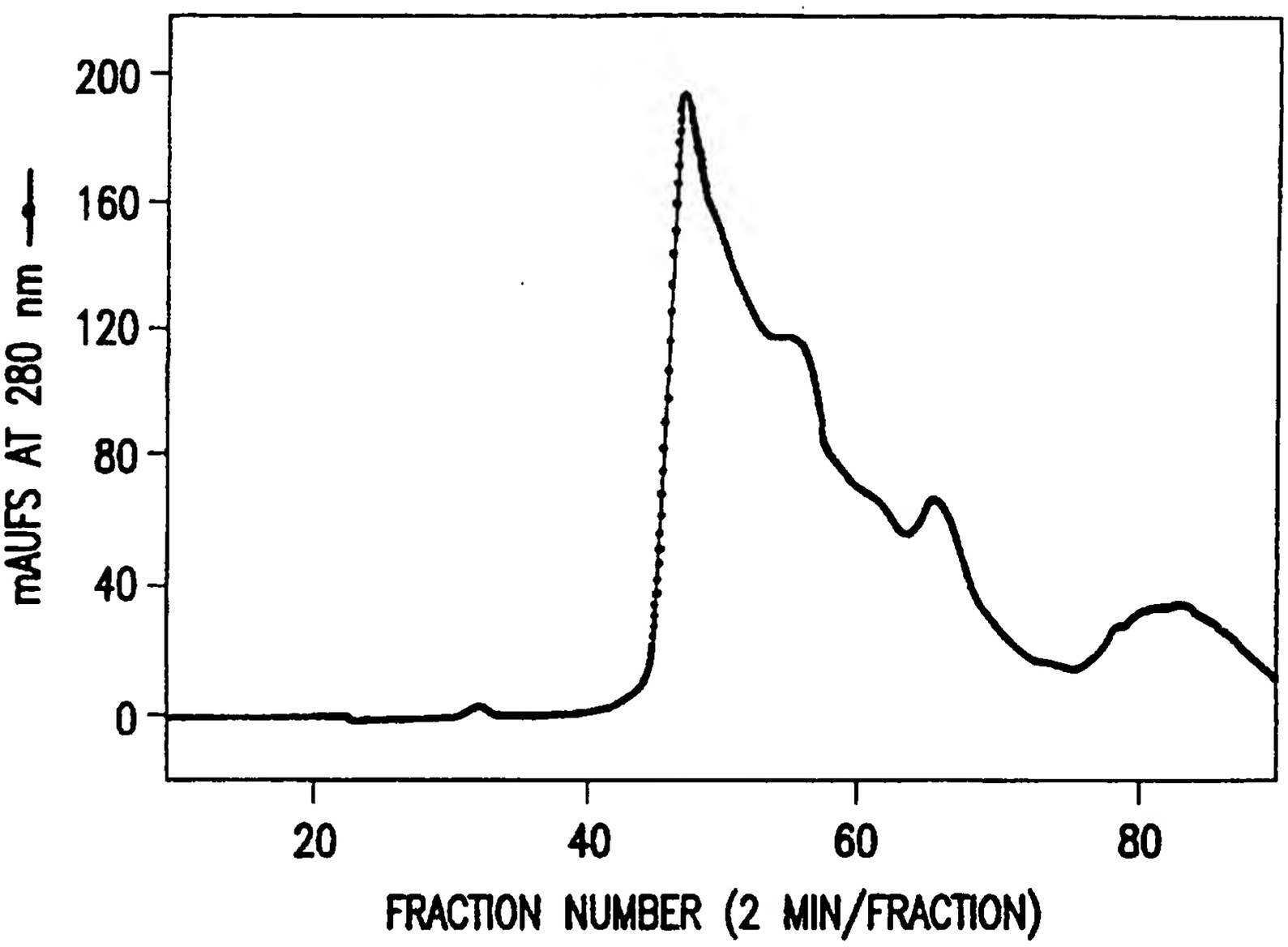
mAUFS AT 280 nm
200
160
120
80
40
0
20
40
60
80
FRACTION NUMBER (2 MIN/FRACTION)

FIG.9A

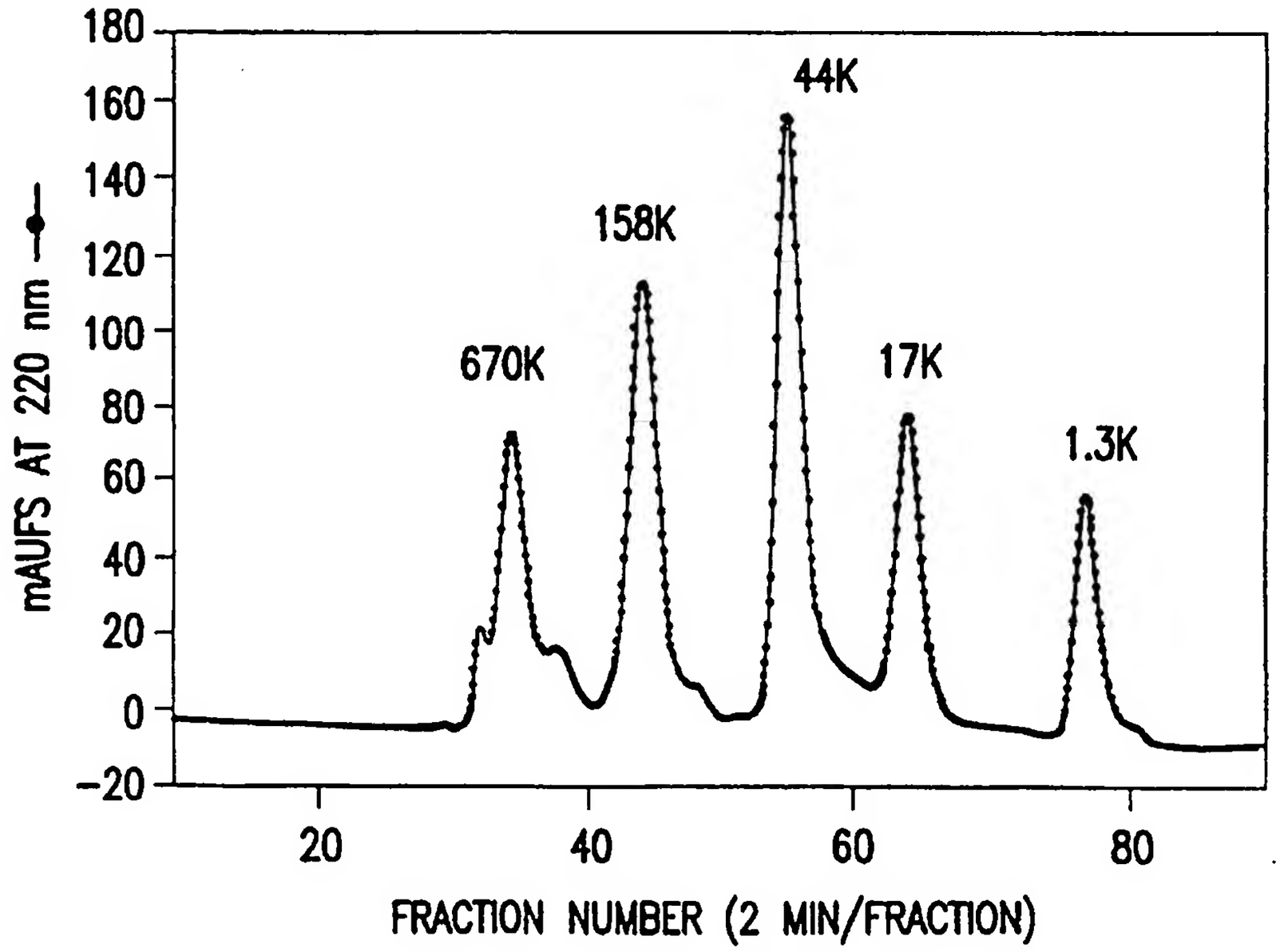
mAUFS AT 220 nm
180
160
140
120
100
80
60
40
20
0
-20
44K
158K
670K
17K
1.3K
20
40
60
80
FRACTION NUMBER (2 MIN/FRACTION)

FIG.9B

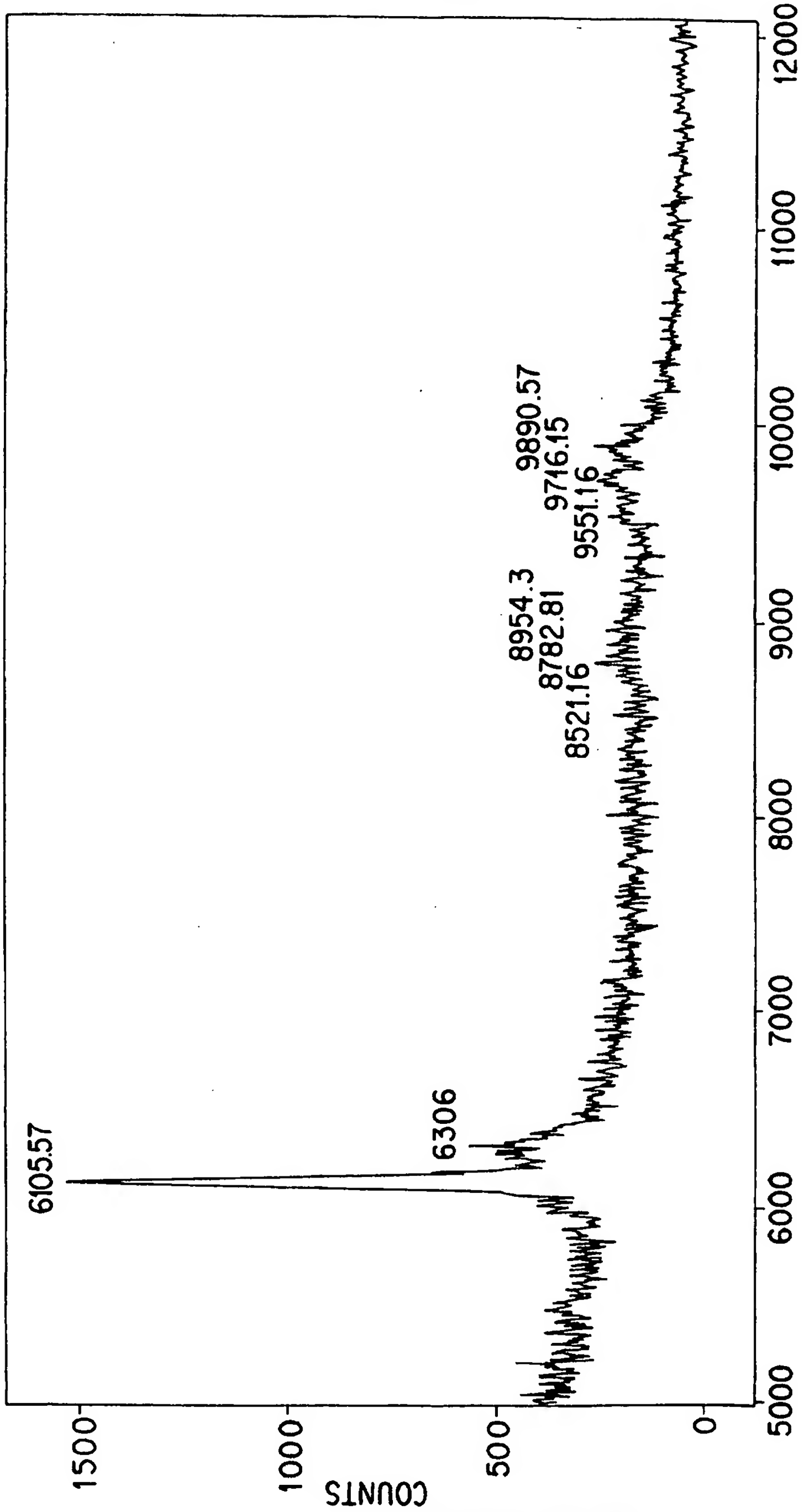
6105.57
6306
8521.16
8782.81
8954.3
9551.16
9716.15
9890.57
COUNTS
1500
1000
500
0
5000
6000
7000
8000
9000
10000
11000
12000
MASS(m/z)

FIG.10A

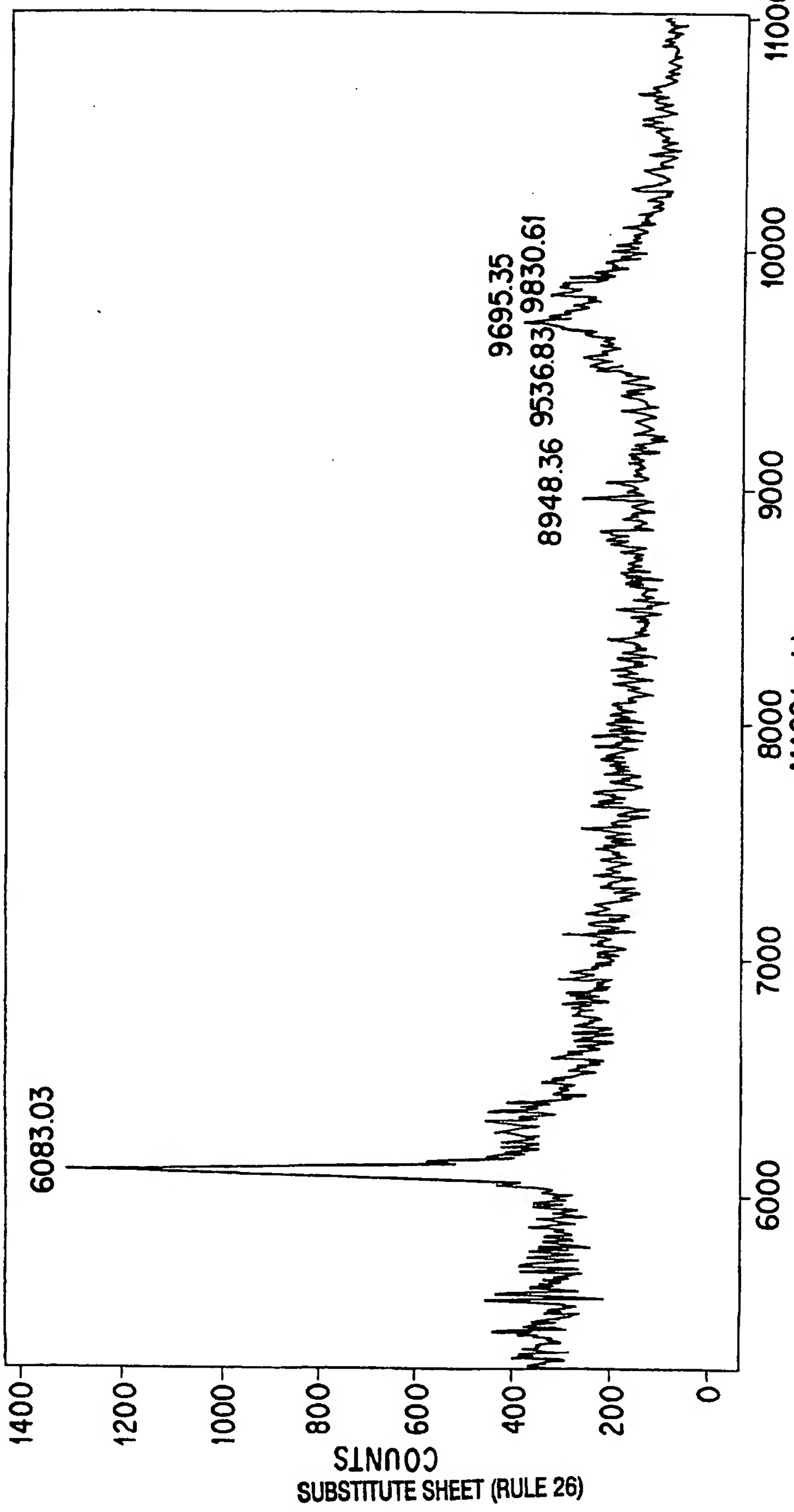
11000
10000
9000
8000
7000
6000
MASS (m/z)
9695.35
9830.61
9536.83
8948.36
6083.03
1400
1200
1000
800
600
400
200
0
COUNTS

FIG.10B

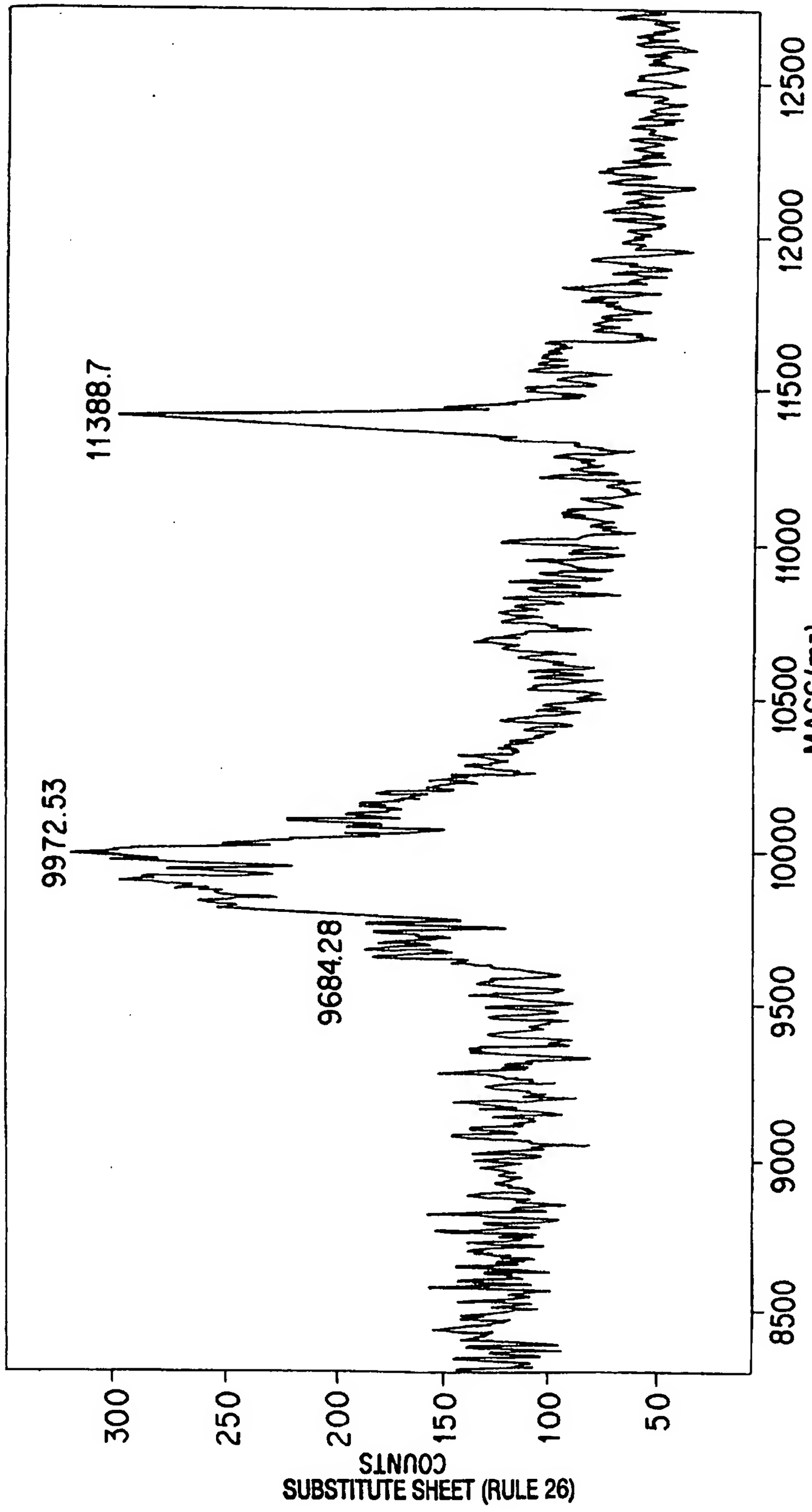
11388.7
9972.53
9684.28
12500
12000
11500
11000
10500
10000
9500
9000
8500
MASS(mz)
300
250
200
150
100
50
COUNTS

FIG.10C

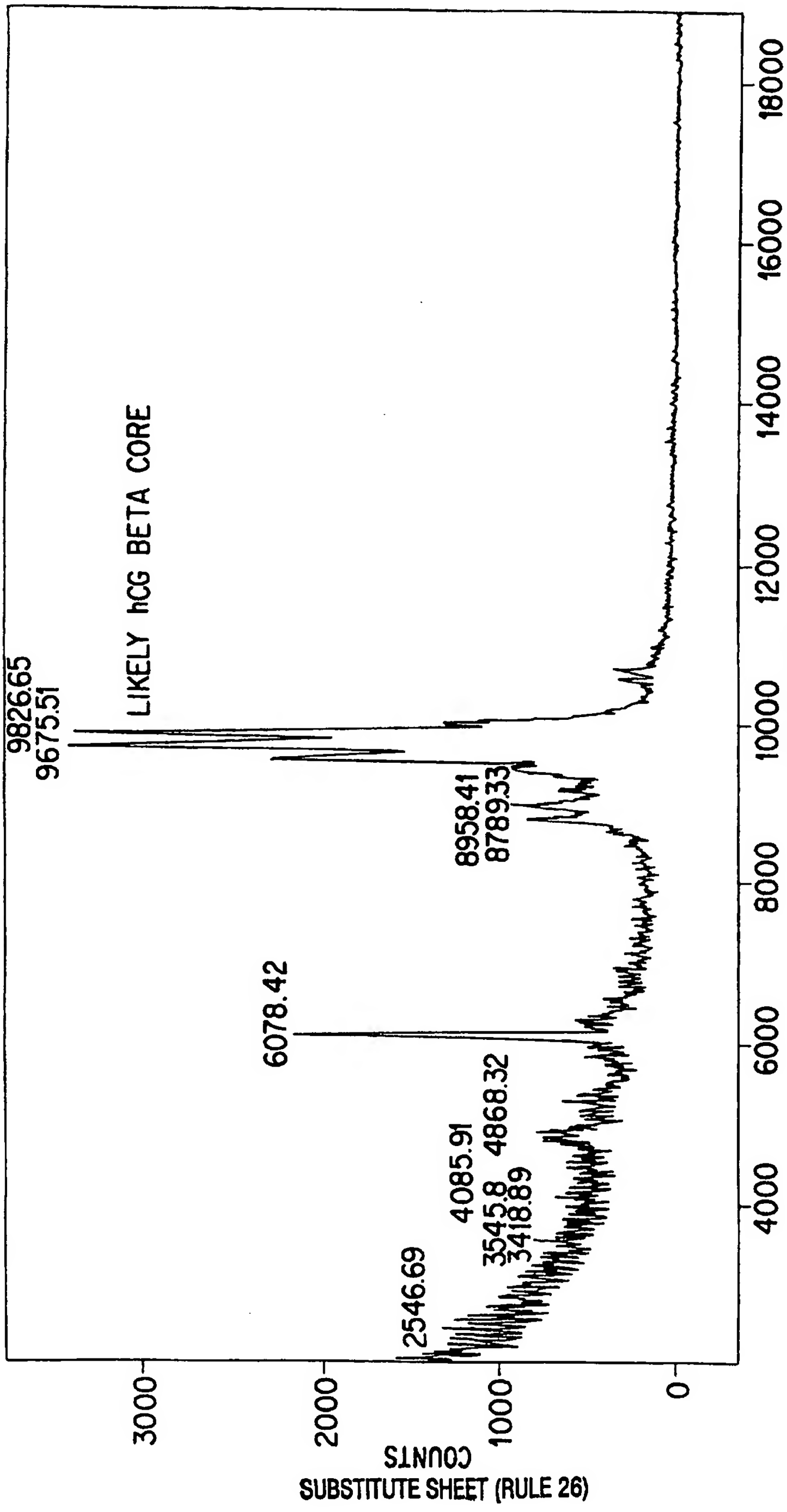
LIKELY hCG BETA CORE
9826.65
9675.51
8958.41
8789.33
6078.42
4085.91
4868.32
3545.8
3418.89
2546.69
COUNTS
MASS(m/z)
3000
2000
1000
0
8000
10000
12000
14000
16000
18000
6000
4000

FIG.10D

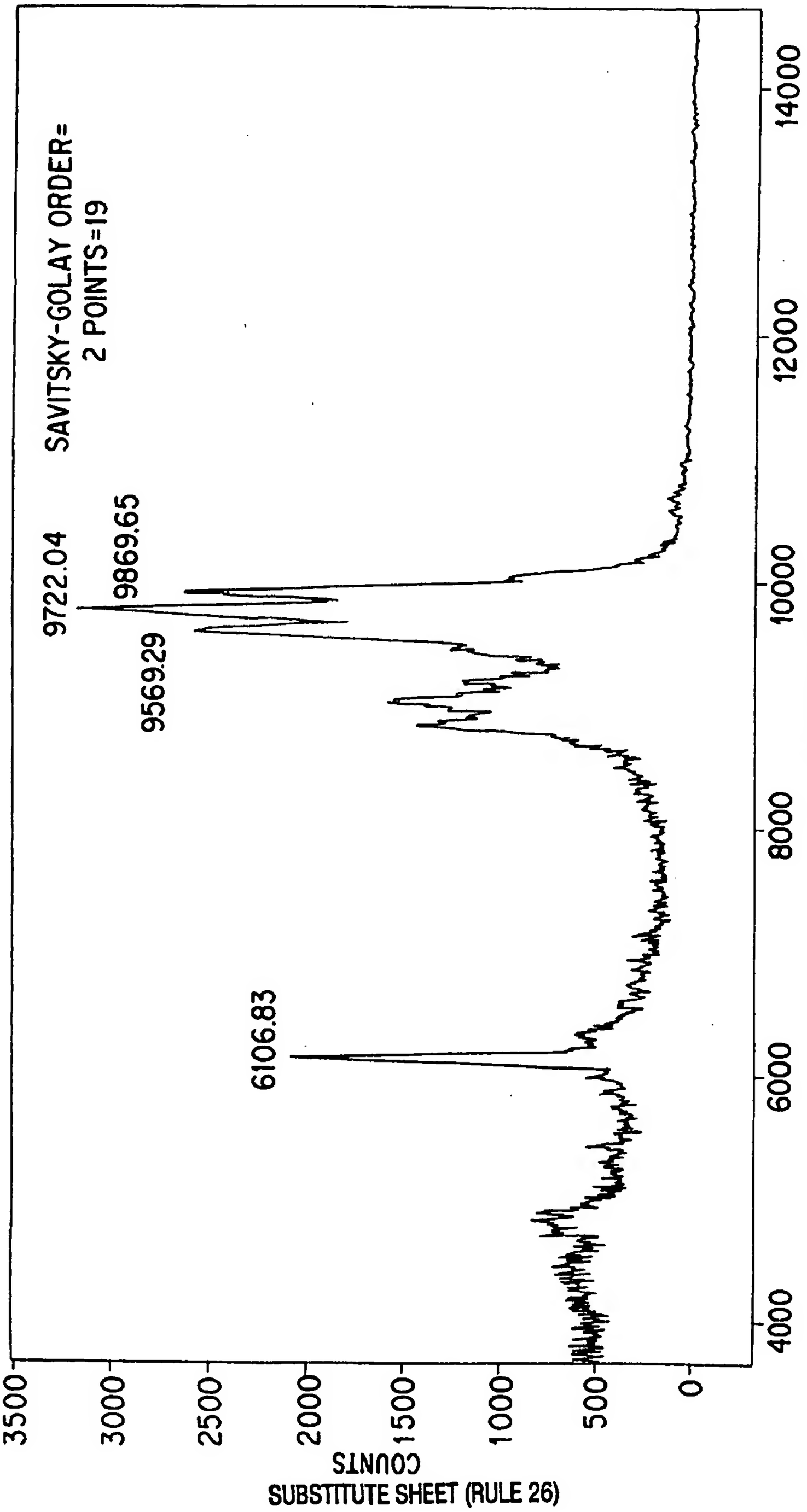
SAVITSKY-GOLAY ORDER=
2 POINTS=19
9722.04
9869.65
9569.29
6106.83
COUNTS
3500
3000
2500
2000
1500
1000
500
0
4000
6000
8000
10000
12000
14000
MASS (m/z)

FIG.10E

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US97/11209

A. CLASSIFICATION OF SUBJECT MATTER

IPC(6) :A61K 38/00; C07K 1/00; C12N 15/00
US CL :Please See Extra Sheet.
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 424/198.1; 435/172.3, 240.2; 514/2, 12-17, 22, 885; 530/350, 412

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

USPAT, JPOABS, EPOABS, CAPLUS, EMBASE, BIOSIS, MEDLINE, WPIDS, FILE REG

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X --- Y	WO 96/04008 A1 (THE GOVERNMENT OF THE UNITED STATES OF AMERICA, represented by THE SECRETARY OF THE DEPARTMENT OF HEALTH AND HUMAN SERVICES) 15 February 1996, see entire document.	1-15, 18, 19, 25-27, 32-36, 38-44, 46, 112-114, 116-124, 131-132 and 136-144 --- 1-27, 30, 32-46, 112-133, 136 and 138-144
Y	US 4,713,366 A (STEVENS) 15 December 1987, see entire document, especially columns 18-28.	1-27, 30, 32-46, 112-133, 136 and 138-144

[X] Further documents are listed in the continuation of Box C. [ ] See patent family annex.

* Special categories of cited documents:

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
10 SEPTEMBER 1997	08 OCT 1997

Name and mailing address of the ISA/US	Authorized officer
Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231	BENNETT CELSA
Facsimile No. (703) 305-3230	Telephone No. (703) 308-0196

Form PCT/ISA/210 (second sheet)(July 1992)★

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US97/11209

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	IYER, K.S.N. et al. Search For Peptide Immunogens Of The β-Subunit Of Human Chorionic Gonadotropin (HCG) Capable Of Eliciting Hormone Specific And Neutralizing Antisera. Int. J. Peptide Protein Res. 1992, Vol. 39, pages 137-144, see abstract, page 138, column 2 and page 140, column 2.	1-27, 30, 32-46, 112-133, 136 and 138-144
Y	DIRNHOFER, S. et al., The Molecular Basis For Epitopes On The Free β-Subunit Of Human Chorionic Gonadotrophin (HCG), Its Carboxyl-Terminal Peptide And The HCGβ-Core Fragment. J. Endocrinology. 1994, Vol. 141, pages 153-162, see entire article.	1-27, 30, 32-46, 112-133, 136 and 138-144
A	US 5,494,899 A (KINCADE ET AL.) 27 February 1996, see entire document.	1-179

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US97/11209

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This international report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

2. ☐ Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

Please See Extra Sheet.

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. ☒ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

☐ The additional search fees were accompanied by the applicant's protest.

☐ No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US97/11209

A. CLASSIFICATION OF SUBJECT MATTER:
US CL :

424/198.1; 435/172.3, 240.2; 514/2, 12-17, 22, 885; 530/350, 412

BOX II. OBSERVATIONS WHERE UNITY OF INVENTION WAS LACKING
This ISA found multiple inventions as follows:

This application contains the following inventions or groups of inventions which are not so linked as to form a single inventive concept under PCT Rule 13.1.

Group I. Claims 1-5 and 112-113 drawn to a composition comprising hCG or beta-hCG for increasing the production of hematopoietic cells and to prevent/treat disorders, classified in class 530, subclass 350+; class 514, subclass 2+; 424, subclass 93.1+.

Group II. Claims 6-9, 14(in part), 15(in part), 16, 18(in part), 19(in part), 17(in part), 18, 21-25, 114-115, 119(in part) and 120(in part) drawn to a composition comprising a hematopoietic cell proliferating protein comprising a portion of the amino acid sequence of beta-hCG to prevent/treat disorders, classified in class 530, subclasses 300-350+ and class 514, subclass 2+, 424, subclass 93.1+, depending upon the size of the protein and selection of amino acids.

Group III. Claims 10-13, 14(in part), 15(in part), 17, 18(in part), 19 (in part) and 121-125 drawn to a composition comprising a hematopoietic cell proliferating protein derivative (e.g. non-class amino acids and D-amino acids) comprising a portion of the amino acid sequence of beta-hCG to prevent/treat disorders, classified in class 530, subclass 300-350+ and class 514, subclass 2+, depending upon the size of the protein, selection of amino acids and the derivitization (e.g. cyclization, glycosylation, amidation, farnesylation etc.

Group IV. Claims 20-24, 30-31 and 126-130 drawn to a composition comprising a cyclic hematopoietic cell proliferating protein comprising a portion of beta-hCG to prevent/treat disorders, classified in class 530 , subclass 300-350+, class 514, subclass 2+, and in other classes depending upon the size of the protein and selection of amino acids.

Group V. Claims 25-29 and 131-135, drawn to a composition comprising a multimeric hematopoietic cell proliferating protein comprising a portion of a beta-hCG analog to prevent/treat disorders, classified in class 530 , subclass 300-350+, class 514, subclass 2+, and in other classes depending upon the size of the protein and selection of amino acids.

Group VI. Claims 30-31 and 136-137, drawn to a composition comprising a cyclic hematopoietic cell proliferating protein comprising a portion of a beta-hCG analog to prevent/treat disorders, classified in class 530 , subclass 300-350+, class 514, subclass 2+, and in other classes depending upon the size of the protein, and the election of amino acids .

Group VII. Claims 32-38 and 116-118 drawn to a composition comprising a hematopoietic cell proliferating homologous and heterologous protein (e.g.fusion protein) comprising a portion of a beta-hCG which lacks contiguous amino acids to prevent/treat disorders, classified in class 530, subclass 300-350+, class 514, subclass 2+,

Group VIII. Claims 39-46 and 138-144 drawn to the use of a composition comprising two different HCG B-CG different purity to prevent or treat disorders, classified in class 530, subclass 350+

Group IX. Claims 47-53 and 145 drawn to the use of a composition comprising HCG or bHCG for in vitro harvesting of a non-recombinant or recombinantly altered hematopoietic cell, classified in Class 530, 514, and 424.

Group X. Claims 54-65 and 146-151 drawn to the use of a composition comprising proteins from B-HCG or in vitro harvesting of a non-recombinant or recombinantly altered hematopoietic cell, classified in Class 530, 514, 424.

Group XI. Claims 66-76 and 152-156 drawn to the use of a composition comprising protein derivatives of B-HCG for in vitro harvesting of a non-recombinant or recombinantly altered hematopoietic cell, classified in Class 530, 514, 424.

Group XII. Claims 77-78 and 157-161 drawn to the use of a composition comprising cyclic protein derivatives of B-HCG for in vitro harvesting of a non-recombinant or recombinantly altered hematopoietic cell, classified in Class 530/514/424.

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US97/11209

Group XIII. Claims 82-86 and 162-166 drawn to the use of a composition comprising a B-hCG analog for in vitro harvesting of a non-recombinant or recombinantly altered hematopoietic cell, classified in Class 530/514/424.

Group XIV. Claims 87-88 and 167-168 drawn to the use of a composition comprising a cyclic B-hCG analog for in vitro harvesting of a non-recombinant or recombinantly altered hematopoietic cell, classified in Class 530/514/424.

Group XV. Claims 89-98 and 169-173 drawn to the use of a composition comprising a b-HCG noncontiguous protein (e.g. fusion protein) for in vitro harvesting of on-recombinant or recombinantly altered hematopoietic cell, classified in Class 530/514/424.

Group XVI. Claims 99-100 and 174-177, drawn to the use of a composition comprising a mixture of different B-HCG compounds of different purity useful for harvesting of a non-recombinant or recombinantly altered hematopoietic cell, classified in Class 530/514/424.

Group XVII. Claims 101-108 and 178-179 drawn to the use of a two-part composition for in vitro harvesting of a non-recombinant or recombinantly he altered hematopoietic cell, classified in Class 530/514/424.

Group XVIII. Claims 109-111 drawn to an assay for pro-hematopoietic activity of a sample comprising hCG or a chain or derivative thereof, classified in class 435, subclass 6+ and class 436, subclass 94+.

Group XIX. Claims 180-181 drawn to utilization of nucleic acid encoding a beta-hCG containing protein and transformed cells in recombinant therapy, classified in class 514, subclass 44+, class 424, subclass 93.1+.

The inventions lack unity of invention, each from the other because of the following reasons:.
Groups I-IX are directed to compositions containing proteins and/or peptides which are structurally diverse and distinct compounds which are capable of separate manufacture and/or use. Additionally, the claims of these groups lack a corresponding technical feature since each drawn to Improper Markush compound groups since the compound claims are completely void of any defined chemical structure which is representative of a common core or nucleus which is essential a common utility. Additionally, the compounds of the Groups encompass peptide and protein compounds which are completely unrelated to one another with regard to amino acid composition and conformation (e.g. cyclic, deletion, substitution analogs, fusion proteins etc.). The in vivo methods are distinct from the assay method since the methods of the different groups utilize different steps to achieve different results (e.g. therapeutic v. diagnostic). Finally, unity of invention only occurs for defined categories of invention (e.g. a first product, its method of making and its use) and does not extend to subsequent methods (e.g. second, third etc) which utilized different steps and/or active ingredients and/or achieve a different purpose.

Because these inventions lack unity of invention for the reasons given above and since:
a. They have acquired a separate status in the art as shown by their different classification;
b. The manual and computer search required for the different groups diverge; and
c. The inventions have acquired a separate status in the art because of their recognized divergent subject matter, restriction for search purposes as indicated is proper.

It is noted by the Examiner, that in the present case, although lack of unity was present, there was insufficient time to permit a request for payment for additional groups. Consequently a general search was performed to the best of the Examiner's ability given the strict time constraints.